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Liquid Fuel Production from Hemicellulose

by

FORINTEK Canada Corp.

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ENFOR Project C-187 (2)

Liquid Fuel Production from Hemicellulose

by

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May 1983

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ABSTRACT

The utilization of wood hemicellulose hydrolyzates by microorganisms for the production of liquid fuels was investigated. Pretreatment of aspen wood by steam-explosion was optimized with respect to maximizing the pentosan yields in the water-soluble fractions of steam-treated substrates. These fractions were then hydrolyzed by dilute sulphuric acid or by the xylanase enzyme(s) present in the culture filtrates of *Trichoderma harzianum* (E58). The relative efficiencies of hydrolysis were compared with respect to the release of reducing sugars and monosaccharides. The hemicellulose hydrolyzates were then used as substrates for fermentation by *Klebsiella pneumoniae* and *Clostridium acetobutylicum* for the production of 2,3-butanediol and acetone-butanol, respectively. When *K. pneumoniae* was grown on glucose or xylose, up to 113 g/L and 88 g/L respectively, of combined butanediol and acetoin could be obtained using acclimatization and fed-batch approaches. Butanediol yields of 0.4-0.5 g per g of sugar consumed was also achieved in cultures grown on hemicellulose hydrolyzates. With *C. acetobutylicum* maximal butanol yields obtained from glucose and xylose were 10.8 g/L and 9.0 g/L, respectively, in the presence of added acetic or butyric acid. When the substrates were wood hemicellulose hydrolyzates, up to 0.16 g butanol could be attained per g of hemicellulose sugar utilized.

GENERAL INTRODUCTION

Recent interest in renewable energy as an alternative or supplement to petroleum-based energy has led to intensive research in the bioconversion of biomass wastes, particularly wood and agricultural residues which are currently cheap, abundant and underutilized (Bisaria and Ghose, 1981; Flickinger, 1980; Rosenberg, 1980). Many promising systems have been developed at the laboratory scale for the production of liquid fuels and chemicals from the cellulose component of the lignocellulosics. Most processes at present, however, cannot compete economically with fossil fuels. It appears certain that the utilization of the hemicellulose portion of the residues, which can account for 20 to 40% of the total biomass carbohydrates (Timell, 1967), can increase the competitiveness of the overall bioconversion process on an industrial scale (Flickinger, 1980). The recent discovery of a pentose-fermenting yeast (Schneider et al, 1981) resulted in renewed interest in the production of ethanol from hemicellulose carbohydrates of agricultural wastes (Gong et al, 1982; Jeffries, 1981), but little emphasis has been placed on wood hemicellulose utilization. In view of the forestry resources available in Canada, we strongly feel that processes for the bioconversion of wood sugars to fuels and chemicals can be feasible in the near future. An integrated approach has therefore been established in our laboratory for the full utilization of the total carbohydrates present in wood. The present study dealt with the production of ethanol, acetone, butanol and butanediol from wood hemicellulose.

I. PRETREATMENT OF WOOD FOR ISOLATION OF HEMICELLULOSES

Introduction

Recent study in this laboratory (Saddler et al, 1982) has demonstrated that pretreatment by steam explosion can render the cellulose fraction of aspen wood chips more susceptible to hydrolysis and subsequent fermentation. Water washing these steam-exploded substrates further facilitated the enzymatic hydrolysis of the cellulose fraction by removing most of the hemicellulose components, resulting in increased accessibility of the cellulose substrate to the cellulase enzymes. Previously little attention has been given to these hemicellulose-rich water-soluble fractions which can also be found in large quantitites as a byproduct of the pulp and paper industry (Herrick and Hergert, 1977). To ensure the economic viability of the liquid fuels from biomass program we consider it paramount that all available biomass carbohydrates be fully utilized. A systematic study was therefore initiated to optimize the steam treatment so as to obtain the maximum amount of pentosans available for subsequent hydrolysis and fermentation.

Materials and Methods:

Steam Treatment of wood substrates: Thin wafers of aspen poplar (25 g) were steam-treated in a high pressure digester (Saddler *et al*, 1982). To impregnate the wood with acid aspen wafers were first evacuated in a suction flask for 1 h and then the acid solution (50 ml) was introduced into the flask through a dropping funnel, allowing the acid to be fully sucked into the wood. Initially aspen wafers, with or without prior soaking in acids, were exposed to saturated steam at 240^{o}C or 250^{o}C for varying durations (15 S to 120 S). The steamed wood was then quantitatively collected, air-dried and weighed. Moistures of representative samples were determined at 105^{o}C for several hours and these values were used for quantitating yields of the steamed wood.

Extraction of hemicellulose from steamed wood: Wood samples after steam-explosion were extracted with water (wood to water, 1:10, w/w) at room temperature for 2h. Portions of these water soluble fractions were further extracted with methanol. Where acid was used in the steaming process the solution was first neutralized with small amounts of NH_4OH and then evaporated to obtain the water-solubles. All of the fractions were analyzed for pentosan content and those with the highest pentosan levels were selected for subsequent hydrolysis and fermentation study.

Analytical methods: Pentosans were assayed by the method outlined in TAPPI standard T.223-05-71. Acid insoluble lignin was assayed by the method outlined in TAPPI standard T222-05-75. Hexosans were analyzed by the anthrone reaction (Shields and Burnett, 1960). Total reducing sugars were analyzed colorimetrically using dinitrosalicylic acid reagent (Miller, 1959). A Varian DMS90 UV-Visible Spectrophotometer equipped with Flow-through cuvettes and interfaced with Apple II Microcomputer was used in the calibration and analysis. A typical standard curve for the DNS analysis of D-glucose and D-xylose was shown in Fig. 1. Individual sugars (monosaccharides and dissaccharides) were analyzed with HPLC. Initially a Varian 500, micropak NH_2-10 column was used with a solvent system of acetonitrile:H_2O (80:20, v/v), a flow rate at 1.5 ml/min and a reflective index (R.I.) detector. Although resolution was satisfactory with cellobiose, glucose and xylose, difficulty was encountered when wood samples were used. The situation was even less satisfactory when UV detector was attempted. Recent use of BioRad HPLC carbohydrate analysis column (300 x 7.8 mm Aminex HPX-87P, heavy metal form) enabled excellent quantitation of monomeric and dimeric sugars at column temperature of 85^{o}C and solvent (water) flow rate at 0.6 ml/ml (equivalent to column pressure at 22 atm). A typical chromatogram for calibration of the sugars using fructose as internal standard was shown in Fig. 2. Analysis of glucose and xylose is linear to 150 g/L. By reducing solvent flow rate to 0.3 ml/min, good separation and quantitation of arabinose, mannose and galactose could also be obtained.

Result and Discussion

In our previous work (Saddler et al, 1982) aspen wood samples had been steam exploded at various temperatures and durations of steaming to determine the optimum conditions for increasing the enzymatic hydrolysis of the cellulose (water-insoluble) component. A parallel study was carried out here to determine the optimum conditions of steam-treatment for maximizing the amount of undegraded hemicellulose in the water soluble fractions (Table 1). By subjecting aspen wood to saturated steam at 240°C for varying duration, we showed that yield of steamed wood and total pentosan (in water solubles and water insolubles) decreased with increase in the duration of steam-treatment. Pentosan yields in the water soluble fractions, however, increased with longer steam-treatment, reaching an optimum of 54.3% at 60 seconds. Further increase in steam-treatment resulted in decrease in the pentosan levels in both the water solubles and water insolubles, presumably due to degradation of the hemicelluloses. Little reducing sugars were released in the water soluble fractions after steam-explosion and no monosaccharides can be detected by HPLC, indicating that the pentosans in the water solubles were still present largely as xylans, but of low molecular weight to render them water soluble.

When methanol extraction was carried out, wood samples after relatively shorter steam-treatment (15-30S) were only partially methanol-soluble, but this solubility increased drastically with samples steam-treated for 60S or longer resulting in almost complete solubilization of the water-solubles in methanol. The exact composition of these fractions and the reason for the different solubilities of the pentosans have not yet been determined. It is possible that the methanol-soluble fractions may contain xylan at even lower molecular weight than the corresponding water-solubles. In general, steam-treatment for 60S or less appeared to be desirable as it represented a compromise in the maximal recovery of pentosans in the water-solubles and the minimal degradation of the pentosans solublized. Alternatively, steam-treatment at 250°C for 15S also resulted in good pentosan yields, but the condition was more difficult to achieve with the present small digester used in the study.

The addition of low levels of H_2SO_4 to wood samples during steam-explosion was then studied for possible improvement in the effectiveness of pretreatment, particularly at lower temperatures or for shorter durations (Table 2). Although the total pentosan yields were not affected, pentosan contents found in water-solubles were increased significantly by acid-soaking of wood. By using a 15 second steam-treatment in the presence of 0.2% and 0.4% H_2SO_4 (based on wood) pentosan levels of 56.2 and 69.5% respectively, of the total pentosan in untreated wood could be obtained, as compared to the 40.9% pentosan attained by similar treatment in the absence of acid. Since a short treatment was used, the extent of degradation was also minimized with over 80% of the total pentosan being recovered after steam-explosion. Prolonged steaming (>30S) in the presence of acid resulted in highly degraded product and a dramatic decrease in pentosan yields. The results clearly demonstrate that the use of dilute acid during the steam-treatment can shorten the duration of steam treatment and improve the effectiveness of the pretreatment process with respect to optimal pentosan recovery in the water-solubles.

Attempts were also made to substitute H_2SO_4 with trifluoroacetic acid (TFA) during the steam-explosion. Although the latter acid was more expensive, it has the advantage of being volatile and could therefore be readily removed by simple evaporation and be recovered by condensing the vapours for subsequent recycling. Using 2% TFA during a 15 second steam treatment yielded good pentosan recovery in the water-solubles, but the levels obtained were generally lower than those obtained from the equivalent treatment with H_2SO_4.

From this study, optimum conditions for maximizing the recovery of undegraded hemicellulose carbohydrates have been established. For the overall bioconversion process of liquid fuel production from hemicellulose to be feasible, it is important that the various steps involved (i.e. pretreatment, hydrolysis, fermentation and recovery) be optimized concurrently. Wood samples showing maximal pentosan contents in water-solubles or methanol-solubles after steam treatment were therefore selected for subsequent hydrolysis and fermentation, as detailed in the following sections.

II. ACID HYDROLYSIS OF STEAM-TREATED WOOD HEMICELLULOSE

Introduction

Since the microorganisms used in the production of acetone-butanol and butanediol from hemicellulose-derived sugars are not capable of direct utilization of carbohydrates in the polymeric form, it becomes necessary to first hydrolyze the hemicellulose component to monomeric sugars (mainly xylose). Most researchers in the field of hemicellulose utilization have focused on the hydrolysis of hemicellulose (or wood) by dilute acids at elevated temperatures (Gong _et_ _al_, 1982; Horwath _et_ _al_, 1983; Lee and McCaskey, 1982; Lee _et_ _al_, 1979; Leonard and Peterson, 1947; Perlman, 1944). Due to its non-crystalline structure and low degree of polymerization (Timell, 1967), hemicellulose can be readily depolymerized to monomeric sugars by dilute acid treatment. During acid hydrolysis of hemicellulose, however, pentose sugars are also rapidly degraded to form furfural (Lee and McCaskey, 1982). To minimize the loss of available sugar for fermentation as well as to prevent the possible accumulation of inhibitors, optimization study on the hydrolysis process has to be carried out, taking into consideration the acid concentration, reaction time, hydrolysis temperature and removal of acids upon completion of hydrolysis.

Materials and Methods:

Preparation of hemicelluloses (xylan). Xylan preparations used in this study were initially obtained commercially from Sigma extracted from larchwood (or oat spelts). Subsequently xylan were prepared from aspen wood (*Populus tremuloides*) using modified methods of Jones *et al* (1961) and Koshijima *et al* (1965). Alcohol-benzene (1:2) extracted aspen, Wiley-milled to pass a 20 mesh screen, was immersed in 24% KOH in an atmosphere of nitorgen at room temperature for 15 hr. The insoluble residues were removed by filtration through a sintered glass funnel and the filtrates were poured into a mixture of ethanol and acetic acid (15:1, v/v) and kept at 0°C. The precipitated hemicellulose was collected and washed with 70% ethanol and 95% ethanol. The resulting product was air-dried and used as standards for hemicellulose hydrolysis studies.

Preparation of holocellulose. Holocellulose was prepared by the method of Wise *et al* (1946). Air-dried wood (aspen) was heated at 70-80°C for 1h in an aqueous solution containing sodium chlorite and glacial acetic acid (1.5 g and 10 drops, respectively, in 160 ml of H_2O). At intervals, the reaction flask was gently rotated. At the end of the first hour, without cooling, 10 drops of glacial acetic acid was added, followed by the addition of 1.5 g of sodium chlorite. The suspension was thoroughly mixed and heating at 70°-80°C was continued for another hour. The process was repeated a third time, then cooled in an ice bath, filtered, washed with ice-water and finally with acetone. The resulting residue is air-dried and used as a model substrate of total wood carbohydrates.

Acid hydrolysis. Optimization of the acid hydrolysis process was carried out with commercial xylan (Sigma) or aspen xylan preparation. Aspen wafers which were untreated or pretreated with steam-explosion were then hydrolyzed using conditions established. Two sets of conditions were tested. Wood substrates were hydrolyzed by 3%-5% of H_2SO_4 (at varying acid to sample ratios) either at 125°C for 1 to 2½h or at 100°C for 2-4h. Residues were filtered off and the filtrates were neutralized either with NH_4OH or with IR45 amberlite resin, filtered, washed and then concentrated under vacuum. The resulting solutions were then analyzed for reducing sugars and monosaccharides, using conditions previously described in section I.

Results and Discussion

The hydrolysis of xylan by dilute H_2SO_4 was investigated so as to maximize the release of xylose while minimizing the loss of sugars due to degradation (Table 3). At 125°C, hydrolysis of xylan by 3% and 5% H_2SO_4 was comparable in its xylose yield. Maximal hydrolysis occurred after a reaction time of 1h. Further increases in the duration of hydrolysis failed to improve xylose yields and may actually lead to a decrease in the final xylose level, presumably due to the breakdown of xylose to furfural. The ratio of acid to sample at 10:1 appeared to provide best hydrolysis efficiency. By decreasing the ratio to 6:1 or 5:1, xylose yield showed a progressive decrease. When the hydrolysis procedure used for breakdown of cellulose was attempted, i.e. 72% H_2SO_4 treatment, the final xylose level was lower than the corresponding values obtained using dilute acid, indicating that a large portion of the sugars hydrolyzed (presumably to completion) had been further degraded. Under optimal conditions, around 57-59% of the theoretical xylose yield was achieved. All the sugars were present as monomeric xylose with no dissaccharides detected.

Wood substrates (water solubles or methanol solubles) showing the highest pentosan yields (Table 1) were then acid hydrolyzed (Table 4). About 65-76% of the total reducing sugars detected in wood hydrolyzates were in the form of monosaccharides. D-xylose was the major component, accounting for over 80% of the total monosaccharides and 34-54% of the samples by weight (about 50-80% of the theoretical yields). Acid-soaking of aspen prior to steam explosion, though effective in increasing the pentosan yield of the wood samples, did not result in enhanced hydrolysis efficiencies by the acid treatment. Although aspen wafers without pretreatment could also be readily hydrolyzed to release hemicellulose sugars, the xylose yield was generally low. Steam treatment followed by water extraction apparently rendered the hemicellulose structure more susceptible to subsequent hydrolysis.

Attempts were then made to improve the final sugar yields by using a lower temperature (100°C) during the hydrolysis reaction (Table 5) so as to minimize sugar degradation. When a 5% xylose solution was first treated as a control for monitoring the degree of sugar loss during the acid treatment, xylose degradation of 12.8% and 31.7% occurred after 2 hours and 4 hours boiling, respectively. The lack of degradation after a shorter duration of hydrolysis, however, was countered by the incompleteness of hydrolysis during the same treatment. This is illustrated by the finding that acid hydrolysis of a 5% xylan preparation for 2 and 4 hours resulted in xylose yields of 64.4% and 64.8% of the original samples, respectively. When the pentosn content of the xylan preparation was taken into consideration, the present procedure resulted in over 80% recovery of the theoretical yield, an improvement over the previous process (125°C for 1h). All the sugars released were found to be xylose with no oligosaccharides detected by HPLC. When a lower acid concentration (1% H_2SO_4) was used, the xylose yield dropped by 32%. No significant improvement was demonstrated by carrying out acid hydrolysis with a higher dilution of substrate (1-2.5% xylan), although a similar treatment with xylose resulted in the recovery of 89% of the original sugar after the treatment. The reason for this apparent discrepancy is at present not determined. Since the prospect of acid hydrolysis in a very dilute broth is unlikely to be economically practical, no further attempt has been made to carry out hydrolysis at lower than 5%-10% substrate levels.

When the water-soluble fractions of steam-treated wood were subjected to the same acid hydrolysis treatment, efficient breakdown of the pentosans occurred (Table 6). Under the conditions tested, duration of steam-treatment did not appear to influence the subsequent hydrolysis with respect to the release of reducing sugars. Based on the reducing sugar analysis by DNS, sugars released from acid hydrolysis were found to approach or even superceded the theoretical maximum obtainable from the pentosan contents of the water-solubles (Table 1). Although it is possible that part of the reducing sugars could arise from the presence of hexoses, it is also likely that natural substrates may contain substances which interfere with the assay systems. When hydrolysis efficiencies were examined basing on xylose release, sugar yields were expectedly lower, but still reflecting an efficiency ranging from 45% to 85% of the theoretical yields. The optimum acid to sample ratio was again 10:1 and less xylose was released when a more diluted sample was used. No attempt has been made at this point to further define the process.

When a similar acid hydrolysis treatment was carried out followed by neutralization with IR 45 resin (Table 7) instead of NH_4OH, the total monosaccharide yields were generally found to be lower (55 to 65% of the theoretical maximum), suggesting that the resins might have removed sugars during the cation exchange. The results also showed that increase in the duration of steam-treatment led to progressively more release of hexosans (or hexoses) into the water-soluble fractions, accounting for up to 10% of the total monosaccharides present. Although no oligosaccharide was detected, the reducing sugar levels remained consistently higher than the total monosaccharides detected. A more in-depth study on the assay systems suitable for natural substrates might be needed to clarify the situation.

As a final test on the effectiveness of acid hydrolysis, the water-insoluble cellulose fractions of steam-exploded wood, holocellulose and aspen wood per sec were subjected to various hydrolysis procedures and analyzed (Table 8). Although a drastic 72% H_2SO_4 treatment was required for effective hydrolysis of hexosans, 3% H_2SO_4 was nevertheless efficient in releasing xylose from the pentosan protions of the samples. Higher xylose yields were actually obtained with the dilute acid treatment presumably due to the lesser extent of sugar degradation under such conditions.

The results obtained from this study clearly demonstrated that dilute acid hydrolysis could be applied for efficient breakdown of wood hemicellulose to release sugars. These sugars, under appropriate conditions, could then be used by microorganisms for fermentation to produce liquid fuels. Since the processes of pretreatment and hydrolysis might result in the formation of byproducts which might be inhibitory to fermentation (such as furfural, hydroxymethylfurfural and phenolics), it is important that each individual condition be tested. The products of acid-hydrolysis obtained from this study were therefore tested for their relative fermentability, as detailed in Section IV and V.

III. ENZYMATIC HYDROLYSIS OF STEAM-TREATED WOOD HEMICELLULOSE

Introduction

Although acid hydrolysis has been shown to be effective in the breakdown of wood hemicellulose to release fermentable sugars, we were interested in the prospect of carrying out the hydrolysis process by enzymes. This latter approach has recently received wide attention in the field of cellulose bioconversion but relatively little emphasis has been placed on it in hemicellulose utilization. Considering that the major sugar component of hemicellulose, xylose, is easily degraded further into furfural and other undesirable products which are potential inhibitors of subsequent fermentations, the alternative approach of using specific depolymerization by enzyme becomes appealing. This will then eliminate the possibilities of sugar loss or inhibitors formation due to degradation of xylose as well as potential pollutant problem associated with the use of corrosive acids. Enzymatic hydrolysis was therefore studied as a direct comparison to the acid hydrolysis process with respect to hydrolysis efficiencies of both methods and the fermentabilities of the sugars derived from either hydrolyzates.

Materials and Methods

Microorganism and media: All of the fungi used were taken from the Forintek culture collection. A spore inoculum was used to initiate growth in shake flasks using Vogels medium (Montenecourt and Eveleigh, 1977). The cultures were grown on 2% solka floc at 28°C and then used to inoculate a 30 L fermentor (New Brunswick Microferm Fermentor, New Brunswick Scientific Co., Inc., Edison, N.J.) with Vogels medium, using 1-2% solka floc as carbon source. The cultures were grown at 28°C for various times and then filtered through a Whatman glass fiber filter. The filtrates were concentrated by rotary evaporation or by membrane filtration with exclusion limit of 10,000 MW (Pellicon Cassette System, Millipore Ltd., Mississauga, Ontario, Canada). Filtrates were assayed for cellulolytic enzymes and stored at 4°C.

Substrates: Xylan standards from larchwood or oat spelts were either purchased from Sigma Chem. Ltd. (St. Louis, Missouri) or prepared from aspen poplar as described in Section II. Wood hemicellulose and cellulose were the water soluble and water insoluble fractions, respectively of steam-exploded wood as described in Section I. Holocellulose preparation was obtained from chlorite treatment of aspen wafer previously described (Section II).

Substrate hydrolysis: Substrates at various concentrations were incubated with enzyme preparations at appropriate dilutions at $50^{o}C$, pH 5.0 or $30^{o}C$, pH 6.5 for different time intervals and assayed for the release of reducing sugars and monosaccharides. Three enzyme preparations obtained from *Trichoderma* E58 were used for initial optimization attempts: original culture filtrates, filtrates concentrated 8 to 10 fold by rotary evaporation and filtrates concentrated 15 to 75 fold with Pellicon membrane filtration. Due to occasional variability in the relative enzymatic activities during culture growth and storage, all enzyme preparations were assayed for cellulolytic activities prior to hydrolysis studies.

Analytical methods: Soluble protein was determined by the method of Lowry *et al* (1951) using bovine serum albumin (Sigma) as standard. Total reducing sugars were assayed by DNS as previously described (Section I). Filter paper activity was determined by the method of Mandels *et al* (1976). Xylanase activity was determined as described previously (Saddler *et al*, 1983) using Sigma xylan (larchwood) as substrate. Carboxymethylcellulase and salicinase activities were assayed under conditions previously established (Saddler *et al*, 1982). Individual sugars were assayed by HPLC as described in Section I. Due to the non-availability of commercial xylobiose for calibration of that sugar quantitatively, xylobiose estimation was performed using the same peak area to sugar concentration factor as xylose. This is based on the finding that glucose and cellobiose also have similar factor (<10% difference).

Results and Discussion

A. Production and properties of the xylanase enzyme(s) from *Trichoderma* sp.

Recent studies in our laboratory have demonstrated an increasing number of fungi with high cellulolytic activities (Saddler, 1982; Saddler *et al*, 1982; Saddler *et al*, 1983). The culture filtrates of such fungi were also shown to possess xylanase activities (Saddler, *et al*, 1983) and consequently capable of hemicellulose hydrolysis. Screening of fungi from Forintek Culture Collection was therefore continued (Table 9). Under the culture conditions used to grow the ten strains of fungi, both cellulase and xylanase activities were detected in the culture filtrates of all the strains tested. The highest activities were again found with *T. harzianum* E58, *T. reesei* C30 and *T. reesei* QM 9414. Since strain E58 was previously successfully used in wood hemicellulose hydrolysis (Saddler *et al*, 1983), efforts were made to improve its enzyme production by optimizing the culture conditions.

When *T. harzianum* E58 was grown on low concentrations of solka floc (1-2%) optimum production of extracellular cellulases and xylanase was detected (Table 10). Xylanase production appeared to reach a peak after 4-5 days and tapered off afterwards, whereas cellulase enzymes required 7-9 days for maximal activity. The production of enzymes by cultures grown on various wood substrates were then investigated (Table 11). When *T. harzianum* E58 was grown on steam-exploded wood, all cellulase and xylanase activities decreased. This is presumably due to the presence of inhibitors in the steam-treated wood substrates. By washing the substrates with water or alkali, the inhibitory effect was removed and all enzyme activities were restored to levels comparable to solka-floc grown cultures. It is at present not determined whether the inhibitory effect observed is directly on the enzyme production or on the activities alone (or a combination of both). When *T. reesei* C30 was similarly treated, xylanase activity in the culture filtrate was low in solka-floc grown cultures, but the cellulase activities appeared to be fully expressed. Growth of *T. reesei* C30 on steam-exploded aspen led to decrease in cellulase activities, in agreement with findings on *T. harzianum* E58. However, xylanase activity increased by 8 fold under the same conditions. Further increases in substrate concentration resulted in a decline of all the activities assayed, presumably due to the higher levels of inhibitors present. By washing the steam-exploded aspen by water and subsequently by alkali, hemicellulose and inhibitors were progressively removed. The finding that xylanase activities in cultures grown on alkali-washed substrate were lower than corresponding cultures grown on water-washed steam-exploded aspen strongly indicated that it is the removal of pentosans, and not inhibitors, which contributed to the decline in xylanase activities, presumably at the level of enzyme production. All cellulase activities on the other hand were similar in both cultures, lending further support to the hypothesis. These results then indicate that *T. reesei* C30 follows "classical" regulation in enzyme production, i.e. cellulase induced by cellulosic substrates and xylanase induced by xylan and hemicelluloses, whereas T. harzianum E58 only produces extracellular cellulases and xylanases when grown on cellulosic substrates.

The stability of the xylanase enzyme(s) during storage was then investigated (Table 12). The xylanase activities remained stable over prolonged storage (30 days) even at $4^{o}C$. No apparent loss of activity was detected through freezing and thawing of the enzyme preparations. By using a concentrated enzyme preparation, there appeared to be a marginal drop in the specific activity of the xylanase enzyme, but it is not determined if the loss could be due to the procedures of concentrating enzymes.

Earlier we have noted that commercial cellobiase exhibits xylanase activity (Table 13) even though the activity was low compared with culture filtrate from T. harzianum E58. However, xylanase enzyme activity does not necessarily reflect the hydrolytic activity of the enzyme system (table 14). Based on the release of reducing sugars during hydrolysis, NOVO cellobiase was actually capable of more efficient breakdown of xylan. There are many factors which may affect the hydrolytic activity of an enzyme preparation, such as the synergism of the enzyme components, the optimum relative concentration of each component, half-life of each component under hydrolysis conditions as well as the reversible and irreversible adsorption of the individual enzyme. It is likely that a combination of these factors may be operative in the present study. The results therefore suggest that the only way to realistically assess hydrolytic potential of an enzyme preparation against a specific substrate is to actually carry out the hydrolysis process under the conditions tested.

B. Enzymatic hydrolysis of wood hemicellulose

Since this study aims at the utilization of aspen wood hemicellulose, we consider it more relevant if we carry out the xylanase enzyme assay on aspen xylan as substrate. A significant difference occurred in the xylanase assay using different xylan preparation (Table 15). The use of aspen xylan as assay substrate should therefore provide a better reflection of the hydrolytic ability of the enzyme on aspen wood fractions.

The optimum conditions for carrying out the enzymatic hydrolysis were then examined (Figs. 3 and 4). Earlier studies had demonstrated that xylanase enzymes were most active at pH 5.0 and 50°C. However, at that temperature, regardless of pH (ranging from 4.5 to 7.0), xylanase activity became very unstable with over 95% loss in activity during the first hour of incubation (Fig. 3). At 30°C or 37°C, the xylanases enzymes were active over a prolonged period. When the patterns of reducing sugars released during hydrolysis under various conditions were compared (Fig. 4), it was found that hydrolysis at 50°C provided a faster initial rate of sugar release, but overall hydrolysis was slightly more efficient at 30°C than the higher temperature, presumably due to the inactivation of enzymes at 50°C. There was nevertheless a further increase in reducing sugar release at 50°C during the second day of hydrolysis. A likely explanation would be the presence of a more thermostable β-xylobiase (β-xylosidase) which would continue to break down dimers of xylose to monomers, resulting in a net increase in reducing sugars. The presence of both xylobiose and xylose as products of hydrolysis lend support to this hypothesis (Fig. 5).

The hydrolysis of wood substrates was then carried out. By concentrating the culture filtrate of Trichoderma E58 by rotary evaporation, a sharp decline in xylanase enzyme activities (specific activities) was noted (Table 16). The enzyme activities were restored by redilution of the enzyme preparation, suggesting that the reversible inhibition was probably due to the presence of fungal metabolites or medium ingredients in the filtrate which became concentrated to an inhibitory level during rotary evaporation. When this enzyme preparation was used for hydrolyzing wood fractions (Table 17), hydrolysis of wood cellulose was generally ineffective. This is in agreement with previous finding (Saddler et al, 1982) that enzymatic hydrolysis of cellulose is effective only after water-wash and alkali treatment. Wood hemicellulose, on the other hand, appeared to be quite readily hydrolyzable by the same enzyme preparation. With the water-soluble fractions of steam-exploded wood, hydrolysis efficiencies were in the range of 59% to 88%, taking into consideration the pentosan content of the water-solubles (table 1).

Attempts were made to further improve the hydrolysis process by using xylan as a model substrate for wood hemicellulose. Three enzyme preparations were tested: the original culture filtrates, filtrates concentrated by rotary evaporation and filtrates concentrated by Pellicon membrane filtration (Table 18). The latter method has the potential advantage of being able to carry out the enzyme concentration faster, with low risk of enzyme inactivation, as well as being able to dialyze away all the low molecular weight fungal medium ingredients or metabolites (also present in the culture filtrates) which may serve as inhibitors of hydrolysis and/or fermentation. The efficiency of the enzymatic hydrolysis of each enzyme preparation was then studied with respect to the release of reducing sugars, xylose and xylobiose under various conditions (Table 19). The original filtrate, as expected, showed the lowest effectiveness in the hydrolysis of xylan. The release of sugars from xylan hydrolysis by the two concentrated filtrates, however, was generally comparable. By carrying out hydrolysis at 30°C, the process was slow, requiring 3-4 days for completion, but final levels of sugars released were comparable to corresponding xylan hydrolysis at 50°C, presumably a reflection of the difference in the relative enzyme stability we earlier observed (Fig. 3).

A more refined study was then carried out to optimize the relative substrate and enzyme levels required for maximal reducing sugar release (Table 20). In general, increase in enzyme level or hydrolysis time resulted in corresponding increase in sugar release from xylan. By using concentrated culture filtrate, efficient enzymatic hydrolysis of xylan at 100 g/L could be accomplished (50-60% of theoretical). Filtrates concentrated by

Pellicon membrane filtration were then used for hydrolysis of steam-explod wood-water-solubles (Table 21). The hydrolysis patterns observed for xylan and water-solubles were similar in most regards. Steam-explosion appeared to be an effective treatment in rendering the wood substrates more susceptible to enzymatic attack. An increase in the duration of steam-treatment resulted in a faster initial hydrolysis under the conditions tested. With prolonged hydrolysis at 30°C, the final outcome of hydrolysis was similar in each case. It is encouraging to note that the efficiencies of enzymatic hydrolysis obtainable are now comparable to acid hydrolysis earlier studied (Table 6).

We next attempted to increase the rate of hydrolysis by using a highly concentrated (75 fold by volume) culture filtrate as a source of xylanase enzymes (Table 22). Using Pellicon membrane filters, xylanase activities of over 12,000 IU/ml could be obtained. An examination of the enzyme activities showed that about 69% of the total xylanase enzyme was lost in the eluant, with about 30% being concentrated as filtrate. The specific activities likewise were about five times higher in the eluants than the filtrates. All other cellulases, on the other hand, had their activities concentrated in the filtrate with little loss in the eluants. The result suggested the presence of a xylanase enzyme with a molecular weight at or below 10,000, the exclusion limit of the membrane filter. When the concentrated filtrates were used to hydrolyze the water-soluble fractions of steam-treated wood, rapid hydrolysis was achieved. Within one day of incubation, the amount of reducing sugars released (Table 23) actually exceeded those accountable by just the pentosan content of the water-solubles. This could be again due to interference in the assay system by the wood substrates or due to the presence of hexose sugars in the water-solubles. Based on the sugar analysis by HPLC, sugar levels released were generally only about 50% of the total reducing sugars. About 18-23% of the sugars appeared to be hexoses (mainly as cellobiose) which is unexpectedly high for the water-solubles. Since a corresponding study on the acid hydrolysis of the same samples revealed the presence of 5-8% of the total sugars to be as hexoses (Table 7), it appeared likely that the "cellobiose" detected could be oligomers of xylose (such as xylotriose or xylotetralose) with similar retention time in HPLC as cellobiose. It will be desirable to obtain oligomers of xylose in pure form for proper identification and quantitation of them. At present no commercial source is available, hampering the progress in this area. Attempts will be made in the future to prepare the incomplete degradation products of xylose polymers so as to define the hydrolysis process.

To prevent the possible accumulation of potential inhibitors inherent in the enzyme and/or substrate preparations due to the high concentration of each component used, we carried out enzymatic hydrolysis at lower enzyme and substrate levels (Table 24). At 2-4% substrate concentrations, hydrolysis occurred readily even with rediluted enzymes (Table 22). The hydrolyzates were then tested for fermentability by _K. pneumoniae_ and _C. acetobutylicum_, together with all previously obtained hydrolyzates. Hydrolysis of the water-insolubles was also carried out to test the possibility of future direct "holocellulose" (i.e. combined cellulose and hemicellulose carbohydrates) utilization. Hydrolysis efficiency of solka floc reached 40-50% (by weight) (Table 25) but wood cellulose after only water-washing was generally poorly hydrolyzed, as we had shown earlier (Table 17). These substrates were also tested for fermentability by microorganisms for the production of fuels and chemicals.

IV. PRODUCTION OF 2,3-BUTANEDIOL (AND ETHANOL) BY *KLEBSIELLA PNEUMONIAE* GROWN ON SUGARS DERIVED FROM WOOD HEMICELLULOSE HYDROLYZATES

Introduction

The production of 2,3-butanediol from hemicellulose derived sugars of wood and agricultural residues has recently been viewed as an alternative approach in the utilization of lignocellulosics for conversion to liquid fuels and chemical feedstocks (Bisaria and Ghose, 1981; Flickinger, 1980; Gong *et al*, 1982; Rosenberg, 1980; Tsao *et al*, 1982). Recent work in our laboratory has successfully demonstrated that *Klebsiella pneumoniae* is an ideal candidate for carrying out the bioconversion process. By the optimization of culture medium and culture conditions, bioconversion efficiency approaching the theoretical maximum has been achieved with organisms grown on sugars known to be present in wood hemicellulose hydrolyzates (Yu and Saddler, 1982a, 1982b). Encouraging results have also been obtained by carrying out fermentations on wood hemicellulose hydrolyzed by acid (Yu *et al*, 1982) and enzymes (Saddler *et al*, 1983). Attempts were therefore made in this study to improve the economic feasibility of the process by maximizing the final butanediol levels in the fermentation broths so as to reduce solvent recovery cost, a major expense in butanediol fermentation (Freeman and Morrison, 1947). A systematic study was also carried out to assess wood hemicellulose substrates after various pretreatment and hydrolysis with respect to their subsequent fermentability by the organisms for the production of butanediol and ethanol.

Materials and Methods

Microorganisms: *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes* NRRL B-199; *Klebsiella oxytoca* ATCC 8724) was obtained from National Research Council of Canada Culture Collection (Strain NRCC 3006). Cultures were maintained on nutrient agar slant at -20°C.

Media: The composition of the chemically defined medium (Kp2) used in this study was shown in Table 26. All ingredients except acetic acid and sugar were prepared together with pH set at 6.5 and sterilized by autoclaving at 121°C for 15 minutes. All sugars were prepared separately in 10 to 40% solutions and were sterilized by autoclaving (121°C, 15 min) or by passage through a 0.22 µm filter (Millipore Ltd., Mississauga, Ontario. Canada). Aliquots of the sugar solutions were then transferred asceptically to presterilized medium for appropriate final concentrations. For studies with initial sugar concentrations above 4%, media were prepared at double strength and then diluted after sterilization with concentrated sugar solutions and sterile distilled water to the desired sugar levels. Acetic acid was prepared as 5% aqueous solution at pH 6.5 and autoclaved separately prior to its addition to the medium (1:9, v/v) before inoculation of bacteria.

Alternatively, PA medium as devised by Jansen (1982) was tested for comparison. The composition of PA medium was listed in Table 27.

Media for aerobic cultures were prepared as 10 ml and 50 ml aliquots in 50 ml and 250 ml Erlenmeyer flasks, respectively, and covered with sponge stoppers. Media for cultures to be grown under finite air supply were prepared in Wheaton serum bottles (10 ml in 60 ml vials or 50 ml in 160 ml vials) and sealed with butyl rubber stoppers and aluminum caps.

Studies with various nutrient supplements or medium modifications were carried out by the addition or deletion of certain medium ingredients before pH adjustment and sterilization. Additions of extra components during fermentation were carried out by transferring small samples of concentrated solutions to the fermentation media to achieve the desired levels of the particular components.

Culture conditions: Working cultures at -20°C was thawed at 37°C for ½ hr and streaked onto nutrient agar plates to be incubated aerobically at 37°C for 24 hr. Colonies were checked for purity and then used to inoculate medium broths (with 1% glucose). These cultures were incubated aerobically at 37°C with shaking (150 rpm) for 24 hr, then used to inoculate fresh test media (routinely with 2% inoculum, v/v) which were incubated at 30°C (150 rpm shaking) under finite air or aerobic conditions for specified time periods. For cultures grown in 50 ml broths in 160 ml vessel, sampling was carried out by withdrawing 2 ml samples periodically for analysis.

Fed-batch system was set up by the daily addition of 2% extra sugars to cultures with various initial sugar levels. The sugar solutions were prepared at 40% concentration in water, Kp 1 medium (i.e. no acetic acid) or Kp 2 medium and filter-sterilized. An appropriate volume was transferred to the medium to achieve the additional 2% level desired. Semi-continuous fed-batch system was carried out by the daily removal of samples from the cultures followed by the addition of concentrated sugar solutions to replace the volume of the removed fluids. Double fed-batch system was set up by the daily addition of sugars together with yeast extract (2% sugar, 0.2% yeast extract).

Acclimatization of cells to high initial sugar concentrations was carried out by serial subculturing of cells grown in media containing increasing concentrations of sugars under finite air condition or aerobic conditions. The optimal scheme subsequently adopted was daily subculturing of _K_. _pneumoniae_ under aerobic conditions through stepwise increase in the levels of glucose or xylose (1%-5%-10%-15% of sugar, using 2% inoculum for each transfer). The resulting cultures were used as inocula in fermentation media with initial sugar levels at 15%.

When wood hemicellulose hydrolyzates were used as substrates for the fermentation process, the hydrolyzates were prepared and autoclaved separately at various amounts. An appropriate aliquot of medium ingredients (prepared at five fold the normal concentration and autoclaved or filter-sterilized) was then added, followed by acetic acid addition whenever required to achieve final acetate levels of 0.3-0.5% and sugar levels of 1.0 to 4.0%. Cultures were incubated at 30°C with shaking (150 rpm) under finite air conditions for various time periods and sampled periodically.

Fermentor studies were carried out using a Bioflo Bench Top Chemostat Model C32 (New Brunswick Scientific Co., Edison, New Jersey) with a working volume of 1.5 L. The fermentor was equipped with pH and D.O. (dissolved oxygen) monitors as well as controls in agitation, aeration, temperature and pH. Medium was routinely prepared and autoclaved separately in three components: concentrated medium ingredients, concentrated sugar solutions and acetic acid solution. The components were then mixed in the fermentor to achieve final sugar level of 5.0% and acetate concentration of 0.5%. Routinely fermentation was carried out at 30°C and agitation rate set at 200 rpm and aeration rate at 0.5 L/min. Sampling was performed at intervals following inoculation (2%, v/v).

All studies with shake flasks were carried out in duplicate. Fermentor runs were repeated at least once for each investigation.

Analytical methods: Culture growth, after appropriate dilution, was monitored by reading optical density at 540 nm (O.D. 540) in a 1-cm light path cuvette with a Spectronic 710 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) or a DMS-90 Spectrophotometer (Varian Associates, Inc., Palo Alta, Calif.). Cells were harvested by centrifuging (4°C) at 10,000 x g for 15 min and the supernatant fluids were analyzed. Alternatively, cultures were harvested with Fisher Microcentrifuge Model 235A (Fisher Scientific Co., Fairlawn, N.J.).

Volatile fermentation products were analyzed by gas chromatography (Ackman, 1972) with a model 4600 gas chromatograph (Varian) equipped with an autosampler and a Varian CDS 401 data system. Chromosorb 101 column (6 ft x 1/8 in., 100/120; Chromatographic Specialties Ltd., Brockville, Ontario) was used with a flame ionization detector. The injection and detector temperatures were set at 160°C and 250°C, respectively. The column oven temperature was operated at 160°C for two min., raised to 190°C in the next min and then held for an additional five min. Helium saturated with formic acid vapor was used as a carrier gas and the flow rate was set at 30 ml/min. Quantitation was performed previously with isobutyric acid or propionic acid as internal standard but the presence of acetyl methyl carbinol (acetoin) in fermentation broths under aerobic conditions resulted in interference of the analysis. At present methanol was used as internal standard and was satisfactory under the conditions used. A typical chromatogram of the standard mixtures used in calibration was shown in Fig. 6.

Reducing sugars and monosaccharides were analyzed by dinitrosalicylic acid reagent and HPLC respectively, as previously described in Section II.

Chemicals: All salts were reagent grade (Fisher Chem. Co., Fairlawn, N.J.). D(+) Cellobiose, MES (2[N-morpholino]-ethanesulfonic acid, D(+) xylose, carboxymethylcellulose, glucuronic acid, galacturonic acid, salicin, xylan and propionic acid were all purchased from Sigma Chem. Co. (St. Louis, Missouri). 2,3-Butanediol was obtained from Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin). 3,5-Dinitrosalicylic acid was purchased from Eastman Kodak Co. (Rochester, N.Y.).

Results

A. Utilization of high sugar concentrations by K. pneumoniae for butanediol production

Recent work in this laboratory has clearly demonstrated the effect of acetic acid addition on cell growth, sugar utilization and solvent production (particularly butanediol production) by *Klebsiella pneumoniae* (Figs. 7 and 8). At an optimal acetic acid concentration of 0.5%, a 2-3 fold increase in the final butanediol concentration was observed. When the acetic acid was added to the medium and autoclaved together, the level of enhancement of the final butanediol concentration was significantly lower than corresponding cultures with acetic acid added after autoclaving of the medium (Fig. 9). The timing of the acetic acid addition in the latter situation did not appear to be critical, since acetic acid added before inoculation, after inoculation (0 h of fermentation) as well as 3h, 6h and 9h after initiation of fermentation all resulted in similar fermentation patterns with respect to butanediol production. Under such conditions, butanediol yields above 90% of the theoretical could be obtained at sugar levels of 4-5%. This encouraged us to study the utilization of higher sugar concentrations (around 200 g/L) so as to obtain the 8-10% final butanediol level to make the product recovery as well as the overall bioconversion process economical.

The effect of initial sugar concentration on butanediol fermentation by *K. pneumoniae* was therefore investigated (Fig. 10). Under finite air conditions and in the presence of added acetic acid, complete utilization of sugars was achieved in cultures grown on 5% D-glucose or D-xylose, resulting in near theoretical butanediol yields (88% and 94% of the theoretical yields from D-glucose and D-xylose, respectively). The bioconversion efficiencies dropped drastically in cultures grown on higher initial sugar levels as reflected both in a decrease in percent sugar utilization and a reduction in butanediol yields. This trend is particularly apparent with D-xylose grown cells. The production of butanediol also decreased drastically when cultures were grown in media with sugars autoclaved together with medium ingredients. Sterilization of sugars (at 10-20%) by membrane filtration, on the other hand, did not significantly affect the fermentation process (Table 28).

A more refined study was then carried out to examine the bioconversion efficiencies at sugar levels of 1-10% (Fig. 11). The results confirmed that sugar utilization and butanediol production from both D-glucose and D-xylose were most efficient at initial sugar concentrations of up to 5%. Similar patterns were observed with the other major sugars known to be present in wood hemicellulose (Table 29).

Several approaches were attempted to improve the butanediol production of cultures grown on high initial sugar concentrations. Since the medium was known to be deficient in nitrogen source (Yu and Saddler, 1982b), various nitrogen supplements were added to the medium. Although significant improvement was noted in butanediol production (Table 30), complete utilization of 10% sugar was not achieved. A fed-batch approach was then studied by the daily addition of D-glucose (Fig. 12) and D-xylose (Fig. 13) to the media during fermentation. To prevent possible dilution of medium ingredients and acetic acid during the addition, the sugars were prepared in water, in (Kp 1) medium without acetic acid and in complete Kp 2 medium (i.e. Kp 1 + HAc). Best results were obtained when the sugars added were accompanied by the complete medium supplements. However, even under such conditions, efficient fermentation took place only during the first three days of incubation. Prolonged incubation with further sugar addition actually resulted in a decline in butanediol levels. An examination of the utilization of sugar during the fed-batch process (Fig. 14) clearly illustrated a slow down in the consumption of sugar during the latter stage of fermentation with a concomittant decline in butanediol production.

Since the addition of further solution during fermentation would invariably lead to an increase in the total volume of liquid in the fermentation vessel, attempts were made to study the fed-batch system under constant volume. Our earlier studies revealed that butanediol fermentation was greatly affected by changes in the ratios of surface area, medium volume and headspace volume of the fermentation vessel. It is therefore desirable to carry out a semi-continuous fed-batch process by the daily removal of samples and then replenish the lost volume by the addition of sugar solutions (Figs. 15 and 16). The results obtained, however, suggest that the failure in this approach was not due to changes in the physical parameters.

A double fed-batch system was then set up by the daily addition of sugar supplemented with yeast extract (Fig. 17). Although sugar utilization under finite air conditions was enhanced during fermentation, production of butanediol remained low. Further improvement in sugar consumption was obtained in a fed-batch process run under aerobic conditions, but butanediol production was generally slow and inefficient.

Efforts were then made to improve the bioconversion process by acclimatizing K. pneumoniae cells to high D-xylose concentrations through serial subculturing in media with stepwise increase in sugar levels (Table 31). The adaptation approach was not successful under finite air conditions and cell "die-off" occurred with repeated subculturing at high sugar concentrations. Since the major objective in an acclimatization process was to enhance the ability of cells to adapt to adverse environmental conditions so as to multiply effectively, we decided to carry out the acclimatization of cells under aerobic conditions to ensure optimal cell growth. A series of experiments was therefore initiated to investigate aerobic fermentation of K. pneumoniae grown on high sugar levels.

Earlier we had shown that at low substrate concentrations (1-2%), little or no solvent production occurred under aerobic conditions, with the majority of products being acetic acid (Yu and Saddler, 1982a). When the organism was grown on higher sugar levels, however, production of butanediol was significant (Table 32). The bioconversion efficiencies were generally much lower than the corresponding cultures grown under finite air conditions. On the other hand, acetoin (acetyl methyl carbinol), a precursor as well as a reoxidized product of butanediol, became a major byproduct. Under aerobic conditions the addition of acetic acid did not appear to enhance butanediol production and may actually inhibit growth and butanediol formation in cultures grown on high xylose concentrations (10%). The reason for this is at present unclear but it is probably due to the production of sufficient quantities of acetic acid under aerobic condition for enzyme induction and activation. Further addition of acetic acid in the medium may then lead to excess acetic acid accumulation which we showed earlier was detrimental to the fermentation process.

The effect of nitrogen supplements to the fermentation media both in the presence and absence of added acetic acid was then tested (Tables 33 and 34). Overall, the addition of yeast extract appeared to be most satisfactory, presumably because it serves as a source of both nitrogen as well as growth factors. The combination of yeast extract and acetic acid seemed to consistently result in higher levels of butanediol accumulation when compared with corresponding cultures with added acetic acid or yeast extract alone. However, all such treatments failed to result in the complete utilization of substrates at concentrations higher than 5%

Acclimatization studies under aerobic conditions were therefore carried out using three different media: Kp 1 medium (Table 35), PA medium (Table 36) and Kp 2 medium supplemented with yeast extract (Table 37). _K. pneumoniae_ cells were shown to adapt readily to stepwise increases in substrate concentration, resulting in faster and better growth as well as improved solvent production. The best performance was again observed in medium with added acetic acid and yeast extract. Using the inocula prepared by various schemes of acclimatization, the effectiveness of the process to the subsequent fermentation of 150 g/L of D-xylose was tested (Table 38). When the final fermentations were carried out in Kp 2 medium with yeast extract under finite air conditions, the effect of acclimatization was not apparent. The same inocula, however, resulted in rigorous fermentation under aerobic conditions, with up to 76 g/L of combined butanediol and acetoin being produced when a stepwise adaptation procedure (D or E) was adopted. Since aerobic respiration may result in conversion of sugars to carbon dioxide and water and not solvents, attempts were made to inhibit respiration by using an electron transport chain inhibitor at the site of the terminal oxidase(s). Such an approach was demonstrated to be effective in Saccharomyces spp. in enhancing ethanol fermentation. By adding low levels of azide, we showed that drastic inhibition of both cell growth and solvent production occurred, suggesting that azide may act at sites other than the terminal oxidase, e.g. in the glycolysis pathway. In any case, this approach was left temporarily until a more thorough study of the metabolic pathway of the organisms could be made.

When the PA medium was used instead (Table 39), the general pattern of fermentation of acclimatized cells were similar to cultures grown under previous conditions. The maximum combined butanediol and acetoin concentration obtainable at the end of fermentation was around 69.6 g/L under aerobic conditions but dropped to around 11.0 g/L under finite air conditions. The results obtained also suggested that sugar utilization based on reducing sugar analysis using dinitrosalicylic acid (DNS) may not be appropriate in situations where high sugar concentrations and/or substances with strong reducing groups (such as azide) are present in the medium.

We next examined the effect of acetic acid, yeast extract or both in aerobic fermentation of _K_. _pneumoniae_ using acclimatized cells as inocula (Fig. 18). The addition of acetic acid alone was again shown to be detrimental to butanediol fermentation. The inclusion of both acetic acid and yeast extract, on the other hand, proved to be the best at enhancing sugar utilization and butanediol production. The use of HPLC for analysis of D-xylose was also shown to be more consistent with the fermentation pattern than the reducing sugar analysis.

When a fed-batch approach was used with acclimatized cells as inocula, rapid consumption of D-glucose (Fig. 19) and D-xylose (Fig. 20) could be observed. The production of butanediol was also rapid and efficient, especially when a high initial sugar concentration (15%) was used. Acetoin was generally produced in significant quantities only at the later stages of fermentation, presumably due to the reoxidation of butanediol upon prolonged incubation. The production of diacetyl and acetic acid remained low throughout the bioconversion process. The patterns were generally similar in a double-fed-batch system in which sugar and yeast extract were added together to the cultures during fermentation (Figs. 21 and 22). The inclusion of yeast extract resulted in further improvements in butanediol production. The effect was again most apparent in cultures with high initial sugar levels. Under the conditions used, 113 g/L and 88 g/L of combined butanediol and acetoin from the consumption of 226 g/L and 190 g/L of D-glucose and D-xylose, respectively, could be obtained within 6 days.

B. Utilization of wood hemicellulose hydrolyzates by K. pneumoniae for butanediol production

Concurrent to our program for enhancing utilization of high sugar concentrations by K. pneumoniae, attempts were made to carry out fermentations on natural wood substrates. The approach used to acclimatize the organism to grow on xylan, in the absence or presence of glucose or xylose, was not successful (Table 40), indicating that the organism does not possess a xylanase enzyme system for direct bioconversion of wood hemicellulose. A parallel study with uronic acids (Table 41) was similarly unsuccessful, although the organism appeared to be capable of butanediol production using the uronic acids as the sole carbon and energy source.

The utilization of wood hemicellulose hydrolyzates was then investigated. Acid hydrolyzates obtained from dilute acid treatment (at 125°C for 1h) (Table 4) were found to be readily fermentable (Table 42), with diol yields of 0.40-0.50 g per g of monosaccharides obtained from the water-solubles of steam-exploded wood. Fermentations were most efficient at low wood sugar concentrations (1-2%) and decreased when higher sugar levels were used. Utilization of acid hydrolyzed aspen wood without pretreatment also suggested that butanediol fermentation could be carried out on wood substrates directly.

The inability of the organism to ferment wood hydrolyzates at higher levels indicated the possible presence of inhibitors in the wood hydrolyzates. Since such potential inhibitors were known to be breakdown products of sugars (e.g. furfural from pentoses and hydroxymethylfurfural from hexoses) or lignin phenolics, attempts were made to lessen the severity of the acid treatment by reducing the reaction temperature to 100°C for 4 hours (Table 5). Again the fermentations were most efficient at low concentration even when acid-treated xylose was used as substrate (Table 43). The butanediol yields were generally lower, however this was due to the expression of values as per g reducing sugars rather than monosaccharides used. When the wood hemicelluloses were fermented after a similar acid hydrolysis procedure (Table 44), only low levels of butanediol were produced, although the reducing sugar consumption was satisfactory (80-90% consumption). By neutralizing the acid hydrolyzates with IR 45 ion exchange resin instead of the routine NH_4OH addition, fermentation on similar substrates was greatly improved (Table 45). Under such conditions, close to 1.0% butanediol production could be obtained from around 2.0% monosaccharides derived from the wood hemicellulose, approaching the theoretical maximum. Fermentation with the water-solubles obtained after a 15 sec steam treatment was found to be less rigorous with regard to sugar utilization and butanediol production than water-solubles from 30 sec and 60 sec steam-treated aspen. The production of butanediol also decreased drastically at higher substrate levels.

When the wood cellulose hydrolyzates were used instead, (Table 46) excellent production of butanediol was achieved even at 4% sugar levels. This was in agreement with previous findings (Saddler _et al_, 1982) that water-washing of steam-treated aspen essentially removed all the soluble inhibitors present in the wood substrates, rendering the cellulose fractions readily fermentable. By using a dilute acid treatment for wood hydrolysis, only the hemicellulose fraction was hydrolyzed (Table 8), resulting in a lower sugar yield. However, butanediol yields of 0.27-0.36 g per g of sugar consumed could still be obtained. Such results clearly demonstrated that both the cellulose and hemicellulose carbohydrates from wood hydrolyzates could be efficiently converted to useful products by _K. pneumoniae_.

A parallel study was carried out with enzymatically hydrolyzed wood substrates to compare the fermentabilities of such substrates with acid hydrolyzates. Efficient bioconversion of holocellulose and xylan was performed by the organism (Table 47), but the levels were generally low. The relatively slow and inefficient fermentation was found to be due to substances present in the enzyme preparation (Fig. 23). By carrying out the fermentation on glucose or xylose in the presence of the _Trichoderma_ culture filtrate (concentrated by rotary evaporation), sugar utilization and diol production were inhibited. When glucose was the substrate, _K. pneumoniae_ could eventually overcome the inhibitory effect, but inhibition persisted in xylose-grown cultures.

Since rotary evaporation could be expected to concentrate all the medium ingredients and fungal metabolites in the fungal culture filtrate, enzyme preparations concentrated by membrane filtration was tested to see if such potential inhibitors could be dialyzed away. When the hydrolyzates of xylan obtained by incubation with various enzyme preparations were compared, no obvious trend in the fermentabilities of the substrates could be observed, (Table 48) except for the product formation in some cases which could be a reflection of the initial concentrations of fermentable sugars available. In this situation, differences in inoculum size apparently was not a major determinant on the outcome of fermentation. The duration of hydrolysis on the other hand appeared to affect the final butanediol level significantly, presumably by making available more xylose monomers for fermentation with prolonged hydrolysis. The pattern was similar when hydrolyzates after three days of incubation of xylan with the enzyme preparations were used as the substrate (Table 49). The adverse effect of the material present in fungal culture filtrates was obvious when high substrate and enzyme levels were used (Table 50). This was clearly shown in cultures grown on 10% xylan hydrolyzed in enzyme preparations concentrated by evaporation, resulting in near complete inhibition of fermentation. By using original culture filtrates or filtrates concentrated by membrane filtration, the inhibition was alleviated, except when high enzyme concentration was used as well. This indicated the possible presence of non-dialyzable inhibitors in the filtrate or could simply be a result of a decrease in the water activity in the fermentation medium. Inhibition of butanediol fermentation by high solute concentrations in culture medium has recently been proposed (Esener, 1981; Jansen, 1982).

Since enzyme concentrated by Pellicon membrane filtration appeared promising because of its high hydrolytic activity (Table 20) and potential fermentability (Table 50), wood hemicellulose was hydrolyzed using this enzyme preparation. The production of butanediol from the hydrolyzates, however, was poor (Table 51). By using a more concentrated enzyme preparation (75 fold concentrated) fermentation virtually stopped even at 5% substrate levels (Table 52). When a rediluted enzyme preparation was used, together with a 5% inoculum, fermentation of the water-solubles could be carried when a 2-4% substrate concentration level was used. These again indicate a combination of solute effect and inhibitors present in the substrate as well as enzyme preparations. No definitive conclusion could be drawn regarding the effect of steam-treatment on fermentation, but results suggest that an increase in pretreatment duration, though enhancing enzyme hydrolysis, could be detrimental to fermentation. This pattern was also observed with the water-insolubles (Table 54). Although efficient bioconversion occurred in all cases, the final butanediol level appeared to decrease with duration of steam-treatment, again suggesting formation of inhibitors in the pretreatment step.

The present study demonstrated that butanediol fermentation could be carried out on wood substrates. Although the substrate levels used at present were still low due to inherent inhibitor problems, the result nevertheless provided valuable insight and background information for the bioconversion of biomass hemicellulose to butanediol.

C. Fermentor study on butanediol fermentation of K. pneumoniae grown on D-xylose

The encouraging results obtained from studies on bioconversion of xylose and wood-hemicellulose-derived sugars prompted us to investigate possible scale-up of the system in a fermentor. Since the fermentor could be run under controlled parameters such as pH, temperature and dissolved oxygen tension (i.e. agitation and aeration), this would enable us to better define the optimum fermentation conditions. The information obtained would also be invaluable for further scale-up in pilot plant or commercial scales.

Due to the time span of this contract, only preliminary results could be accomplished at this point, but data accumulated so far strongly indicate that our study in shake-flask cultures could be extrapolated to a fermentor system. When the fermentor was run for butanediol production with the organism grown in Kp 2 medium with slow aeration rate (Fig. 24), high levels of butanediol were produced in 3 days, reaching 18 g/L from the consumption of 50 g/L of xylose. Under such conditions, acetoin production was significant, particularly with prolonged fermentation. The pattern of fermentation and solvent yields were therefore directly comparable to shake-flask studies carried out earlier (Section A). During the fermentation, dissolved oxygen tension decreased steadily with cell growth and became undetected throughout the period of solvent production. At later stages of fermentation when sugar consumption was complete, the dissolved oxygen level began to rise slowly concomittant to the reoxidation of butanediol to acetoin. The pH of the fermentation medium, on the other hand, declined slowly at the onset of fermentation from 6.5 to 6.2, but never dropped to below 6.0. This could be due to the presence of acetic acid in the medium which appeared to inhibit acid formation during fermentation. Together with the use of MES buffer, the entire fermentation could be carried out without using pH titration.

When the fermentation was carried out in a closed system, without aeration (finite air system) (Fig. 25), there was a long lag period (∿4 days) prior to onset of sugar utilization and solvent production. Complete consumption of a 5% sugar solution was achieved in 8 days, with butanediol levels reaching 16.0 g/L The acetoin level remained low throughout the fermentation process.

By increasing the medium volume in the fermentor from 1 L to 1.5 L, fermentation was slower and less efficient (Fig. 26). Although sugar utilization was completed after 5 days, the highest diol level attained was only around 12.0 g/L. The effect of medium surface area, medium volume and headspace volume and their relative ratios have previously been shown to exert a profound influence on the production of butanediol in shake-flask cultures. Apparently a similar phenomenon is operative in the fermentor as well.

Attempts were then made to improve the economic feasibility of the system as well as to simplify the medium by the deletion of MES, a relatively expensive buffer and consequently impractical for industrial use. The removal of MES appeared to shorten the lag period shown earlier (Fig. 27 vs 26). Sugar utilization was rapid and complete, but final butanediol levels were below 10 g/L.

Since the pH of the medium remained above 6.0 during fermentation, it appeared that such decreased production of butanediol was not due to the effect of pH. It is not known at present what other role MES may have in butanediol fermentation. It will be of interest to study this in more detail especially since the requirement for buffer is now eliminated by the presence of added acetic acid. The prospect of running butanediol fermentation without need for buffer or automatic pH control will certainly be a distinct advantage for industrial applicability.

Discussion

For a bioconversion process utilizing lignocellulosic residues to be successfully applied in industrial fermentation, the most crucial criterion is the yield of useful products obtainable on a weight to weight basis from the substrate consumed (Flickinger, 1980). In our current effort to produce liquid fuels and chemical feedstocks from hemicellulose hydrolyzates we have worked towards maximizing the yield of butanediol while attempting to reduce the production of acetic acid (Yu and Saddler, 1982a, b). This has been accomplished by carrying out the fermentation process under finite air conditions and in the presence of added acetic acid (Yu and Saddler, 1982b). By a combined mechanism of induction and activation by acetate on the enzymes involved in butanediol metabolism (Johansen et al, 1975), butanediol yields of 84% and 94% of the theoretical yield could be obtained from D-glucose and D-xylose, respectively, at a 5% sugar concentration. Production of acids was virtually eliminated and over 95% of the added acetic acid was actually consumed, presumably by its condensation with pyruvate for additional production of butanediol (Reynolds et al, 1937; Stahyl and Werkman, 1942). Such encouraging results prompted us to further investigate the process for its applicability to the bioconversion of higher sugar concentrations. This approach is particularly important in butanediol fermentation due to the high boiling point of butanediol ($\sim 180^oC$). By carrying out fermentation at high substrate levels, one can then maximize the final butanediol concentration in the fermentation broths and thereby reduce the cost of the highly energy intensive and expensive process required for product recovery (Freeman and Morrison, 1947; Rosenberg, 1980). For the same reason we fermentation over continuous fermentation, even though the latter can probably result in better productivity (g product/L/h) (Pirt and Callow, 1959).

Earlier workers had noted that high initial substrate concentration were inhibitory to butanediol fermentation (Ledingham and Neish, 1954; Long and Patrick, 1963) presumably due to the effect of decreased water activity (i.e. increase in solute concentration) on the organism (Esener et al, 1980; Jansen, 1982). Nevertheless, they were able to successfully demonstrate the fermentation of 5 to 15% of substrates by K. pneumoniae in complex media containing a wide variety of raw materials (Freeman, 1947; Ledingham and Neish, 1954; Long and Patrick, 1961; 1963; McCall and Georgi, 1954; Olson and Johnson, 1948; Perlman, 1944). Our present attempt to carry out fermentation at high sugar levels in a chemically defined medium was not successful under finite air conditions, resulting in a decline in cell growth, percent sugar utilization and bioconversion efficiency. The inhibition on butanediol production was not as apparent when fermentation was carried out under aerobic conditions even when unacclimatized cultures were used. This suggested that the substrate level itself probably was not directly responsible for the observed effect. A more likely explanation appeared to lie in the basic difference in the two systems of fermentation.

Bioconversion under finite air conditions, being a closed system could lead to build up of gaseous end products (such as carbon dioxide) and/or gas pressure in the vessels which could then inhibit further cell growth, sugar utilization and butanediol production. Alternatively, the inhibitory effect observed could be due to an increase in oxygen demand required for the bioconversion of high substrate concentrations. Consequently under finite air conditions, with a limited supply of oxygen, fermentation was not able to proceed to completion. At present we still have not defined which of the mechanisms may account for the inhibitory effect observed under finite air conditions.

However, by combining the approaches of acclimatization and fed-batch, we are now able to efficiently utilize high sugar concentrations to produce butanediol. It has been estimated that 200-250 g/L of sugar would have to be utilized in order to reach the final butanediol level (around 80-100 g/L) required for the economical recovery of the product (Jansen, 1982). By the inclusion of acetic acid and yeast extract in the medium, we have reached the high butanediol level desired and at a high bioconversion efficiency, surpassing all previously published yields (Rosenberg, 1980).

The present study also demonstrated the capability of _K. pneumoniae_ to utilize sugars derived from wood hemicellulose after acid or enzymic hydrolysis. At low substrate levels (around 2%), butanediol production was close to the theoretical maximum. The bioconversion efficiency declined considerably with higher substrate concentrations, presumably due to the presence of inhibitors accompanying the substrate as a result of steam-explosion and/or hydrolysis. When dilute acid hydrolysis was carried out followed by neutralization with IR 45 resins, the fermentability of the substrates was improved, compared to corresponding substrates neutralized by NH_4OH. It appeared likely that the IR 45 treatment resulted in the partial removal of certain inhibitors of fermentation, but the identity of such substance(s) was at present undefined. The wood hemicellulose after enzymatic hydrolysis posed even greater problems to subsequent fermentation than acid hydrolyzates. Under such conditions, the crude enzyme preparation appeared to contain inhibitors to cell growth and/or butanediol production. Although the use of enzymes after membrane filtration improved the fermentability of the system, high enzyme concentrations continued to remain inhibitory. The use of original culture filtrate in an unconcentrated form was favored for fermentation, but the low hydrolysis efficiency resulted in lower levels of available sugars for the production of solvents. The data accumulated therefore suggested that effective bioconversion of natural substrates could be attained only with low levels of substrates or enzyme. Earlier workers (Perlman, 1944) have overcome such inhibitor problems in wood substrate by acclimzatizing the organism to the substrate. Attempts in this area could prove useful in our studies as well.

V. ANAEROBIC PRODUCTION OF LIQUID FUELS FROM HEMICELLULOSE

Introduction

In our earlier work to produce liquid fuels and chemical feedstocks from sugars derived from wood hemicellulose, we have studied *Clostridium thermohydrosulfuricum*, *C. thermosaccharolyticum* and *C. acetobutylicum* for their reported ability to carry out bioconversion of D-xylose to liquid fuels (ethanol, acetone and butanol) (Flickinger, 1980; Rosenberg, 1980; Wang *et al*, 1979; Wiegel *et al*, 1979). Although good solvent yields could be achieved with *C. acetobutylicum* grown on D-glucose, D-mannose and D-cellobiose (Mes-Hartree and Saddler, 1982), production of acetone and butanol was inefficient in cultures grown on D-xylose. Since D-xylose is a major component of hemicellulose (Timell, 1967), it is essential that this sugar be readily utilized if the conversion of hemicellulose to butanol is to be fully efficient. Extensive studies were therefore carried out in this work to optimize the conversion of D-xylose to useful solvents. The utilization of sugars derived from hemicellulose hydrolyzed by acid or enzymes was also investigated.

Materials and Methods:

Microorganism: C. thermosaccharolyticum (ATCC strain 25773 and 7956) and C. acetobutylicum (ATCC 824 and 2490) were obtained directly from ATCC (American Type Culture Collection). Cultures were routinely maintained in 10 ml of reinforced Clostridia medium (Oxoid) in 60 ml serum vials containing $N_2:CO_2$ (80:20, v/v) or 100% H_2 headspace gases (Miller and Wolin, 1974).

Culture conditions: Medium for C. thermosaccharolyticum was modified from Wang and Fan (1981). Medium was prepared anaerobically using Hungate's technique (1950) as modified by Miller and Wolin (1974) using O_2-free gases (Linde Specialty Gases, Union Carbide Canada Ltd., Toronto, Ontario) of nitrogen in the headspace. Cultures were inoculated (5%, v/v) and incubated at 60°C under stationary conditions for 2 days.

Medium (CAB) for growth of C. acetobutylicum was modified from Wang et al (1977), containing 0.75 g/L of KH_2PO_4; 0.75 g/L of $K_2HPO_4.3H_2O$, 2.0 g/L of $(NH_4)_2SO_4$, 0.20 g/L of $MgSO_4.4H_2O$, 10 mg/L of $MnSO_4.7H_2O_4$, 10 mg/L of $FeSO_4.7H_2O$, 1.0 g/L of NaCl, 5.0 g/L of yeast extract and 0.50 g/L of cysteine-hydrochloride (Mes-Hartree and Saddler, 1982). Cultures were prepared under strict anaerobic conditions and, unless otherwise stated, 100% H_2 was used as head space gas. All sugar solutions (40%, w/v, except for D-cellobiose at 10% concentration) and acids (5%, w/v) were similarly prepared, sterilized and then transferred anaerobically to presterilized media to give desired final levels. Cysteine-hydrochloride was prepared anaerobically as a 1% solution (w/v, pH 6.5), sterilized and then added to media at various concentrations prior to inoculation. In all subsequent studies, cys-HCl has been deleted from the medium unless otherwise specified. Incubations were carried out at 37°C under stationary conditions.

Substrates (wood hemicellulose hydrolyzates) were also prepared and autoclaved separately as the medium and then mixed with medium to give various sugar concentrations prior to inoculation. All studies were carried out in triplicate.

Analytical Methods: Growth of the organism was monitored by reading culture optical density at 660 nm wavelength. Cells were harvested by centrifuging (4°C) at 10,000 x g for 15 min and the supernatant fluids were analyzed for volatile fermentation products by gas chromatography as previously described (Section IV). Quantitation was earlier performed using isobutyric acid as internal standard, but the production of acetyl methyl carbinol by *C. acetobutylicum* under certain conditions led to interference in the assay system due to the similar retention times of the two compounds. Methanol was therefore selected for the present system and was satisfactory under the conditions tested. A typical chromatogram of the standard mixtures used in calibration was shown in Fig. 28.

Reducing sugars and individual sugars were analyzed by DNS assay and HPLC analysis, respectively, as described in Section I.

Result

Earlier work in this laboratory had showed that *C. thermosaccharolyticum* could utilize sugars present in wood hemicellulose for ethanol production, but the solvent yields were generally low. Efforts were therefore made in this study to improve the bioconversion process by further modification of the medium used. When cultures were grown in a variety of nitrogen sources and reducing agents, it was found that $(NH_4)_2SO_4$ and thioglycollate were the best combination for optimal ethanol production with strain 7956, whereas strain 25773 required urea and cysteine for best performance (Table 55). Solvent production by the better solvent producer (strain 7956) was then carried out on major sugars known to be present in wood hemicellulose hydrolyzates. Although fermentation took place with all sugars tested, ethanol production was uniformly low with the majority of the products being acids (Table 56). By preparing sugars separately so as to prevent formation of inhibitors during autoclaving of sugars in the presence of medium ingredients (Table 57) we failed to improve the fermentation process. When wood hemicellulose hydrolyzates were tested (Table 58), low levels of ethanol were attained even with prolonged incubation, casting doubt on the suitability of this organism for industrial production of ethanol either in mono- or co-culture systems.

We next turned our attention to acetone and butanol production (ABE fermentation) by C. acetobutylicum which has been shown to produce solvents efficiently with D-glucose, D-mannose, D-cellobiose and L-arabinose (Table 59). Since the solvent levels from high substrate concentrations (5-10%) were generally low, attempts were made to enhance solvent production by preparing sugars separately and then mixed with medium ingredients after autoclaving. However no significant improvement in butanol production with the possible exceptions of D-glucose and D-cellobiose, was obtained, suggesting that incomplete fermentation was not due to the destruction of sugars and/or the formation of inhibitors during the autoclaving of sugars with medium components.

We then tested the effects of added buffer on the fermentation process. Earlier we had studied the influence of $CaCO_3$ on butanol production. Although sugar utilization was stimulated with increasing $CaCO_3$ concentration, butanol production was actually inhibited, possibly by the accumulation of CO_2 from the breakdown of $CaCO_3$ in solution. We therefore used MES, an organic buffer, instead (Table 60). The addition of MES in ABE fermentation was found to be detrimental to sugar utilization as well as solvent production. Further attempts to improve butanol production by acclimatization were also unsuccessful (Table 61) and eventually led to "die-off" of the cultures in all cases. Growth and solvent production of cells grown on wood hemicellulose hydrolyzates (Table 62) were poor, even with prolonged incubation. No improvement in solvent production was achieved by the addition of high ferrous ion concentration in the medium (Table 63). The influence of the inoculum age (Table 64) or head space flushing and/or pressurizing (Table 65) was also not apparent with respect to butanol production.

The effect of cysteine concentration on the solvent production of C. acetobutylicum was then studied (Fig. 29). The presence of cysteine in the medium at concentrations above 0.25 g/L resulted in a significant decrease in the production of solvents from cultures grown on either glucose or xylose. When cysteine was deleted from the medium while maintaining an anaerobic environment by gassing with hydrogen, butanol yields from growth on xylose were almost three times higher than the levels attained in the presence of cysteine above 0.5 g/L concentrations. The pattern was similar with glucose-grown cells. When the initial pH of the medium was brought up to 7.0, a further enhancement in solvent production was obtained with glucose-grown cells (Fig. 30) but had no apparent influence on the solvent production of xylose-grown cells except for the accumulation of acids at higher pH (Fig. 31).

Attempts were then made to improve butanol yields by modifying the inoculum preparation. The nature of the inoculum affected the fermentation process significantly (Tables 66-71). When the inoculum was grown in medium in the presence of cysteine, a 2 day inoculum culture subcultured from 2 week stock (in RCM) resulted in maximal solvent production from xylose (Table 66), with up to 4.7 g/L of butanol being produced. By deleting cysteine in the inoculum medium, the influence of stock culture and inoculum culture ages on the subsequent acetone-butanol production was not apparent (Table 67). Acclimatization of the organism by using xylose-grown inoculum did not result in improved solvent yield as expected (Table 68). Cell growth, sugar utilization and solvent production all decreased with this treatment, presumably due to a lower cell number in xylose-grown cultures. Previously it was shown that refrigeration of the medium led to decreased solvent production, possibly as a result of air entering into the vials during the cooling process. By pressurizing the prepared medium vessels with hydrogen prior to storage, such detrimental effects were removed (Table 69).

Heat-shocking of C. acetobutylicum was reported to be crucial for good fermentation (Spivey, 1978) and did occassionally improve solvent production slightly (Table 70). However, with inoculum grown in medium in the presence of cysteine, such a treatment was deleterious and at times failed to initiate fermentation (Table 71). In view of the various modifications adopted by researchers in the heat shock treatment (e.g. Prescott and Dunn, 1959; Spivey, 1978), a more detailed study is definitely necessary before reliable fermentation can be achieved. By carrying out fermentation at 30°C, a temperature considered to be optimal under certain conditions (Maddox, 1980), butanol production from glucose grown cells was improved, but butanol yield from xylose-grown cultures actually declined (Table 72).

Under the present culture conditions, solvent production by C. acetobutylicum grown on all major sugars present in wood hemicellulose, except D-galactose (Table 73) has been significantly improved over previous yields (Mes-Hartree and Saddler, 1982). Butanol production was also observed by cultures grown on D-glucuronic acid as the sole substrate. Solvent production was still poor however when D-xylose was used as the substrate, with most of the products being in the form of acids. Recently it has been shown that acetone and butanol production by C. acetobutylicum can be induced by the addition of acetate and butyrate to the medium (Gottschal and Morris, 1981). Bahl et al (1982) also reported a shift in the fermentation from acid to solvent production when butyrate was added to the cells in continuous cultures grown on glucose. This approach was therefore tested here for possible enhancement in solvent production from xylose.

When acetate was added to glucose-grown cultures, only minor improvements in solvent yields were obtained regardless of the time of acid addition (Fig. 32). With xylose-grown cultures, on the other hand, solvent production was enhanced by the addition of 0.5-1.0 g/L of acetic acid to the medium (Fig. 33), although inhibition occurred after higher levels of acetic acid was added. The stimulatory effect was most apparent when the acid addition occurred prior to fermentation (Fig. 33a) compared to addition after 1 day of growth (Fig. 33b). Highest acetone and butanol levels were obtained when a 1.0 g/L acetic acid concentration was used, resulting in values which were 3-4 times higher than those found in the control without added acetic acid. The patterns were followed closely by cell growth and sugar utilization (Fig. 34).

When butyric acid was added instead of acetic acid, the general enhancement was similar. No major improvement was observed with glucose-grown cultures (Fig. 35). With xylose-grown cells, the stimulatory effect was observed only if the addition was made prior to inoculation (Fig. 36a). Under these conditions, cell growth, sugar utilization (Fig. 37) and solvent production (Fig. 36) all increased with an increase in butyrate concentration. Addition of acid after one day of growth did not result in any significant increase in the fermentation products and was actually inhibitory at higher butyric acid levels (Fig. 36b).

The utilization of wood hemicellulose hydrolyzates by C. acetobutylicum was then studied. When the acid hydrolyzates of steam-exploded aspen water-solubles were used as substrates, the production of solvents were uniformly low (Table 74), suggesting that the organism is very susceptible to certain inhibitors in the substrates. The bioconversion of acid hydrolyzed wood cellulose, on the other hand, was more promising (Table 75) resulting in near completion of the fermentation process and a final butanol yield of up to 9 g/L, close to theoretical yield. When wood hemicellulose obtained by dilute acid treatment of aspen and holocellulose (Table 8) was used as substrate, fermentation also occurred readily giving a good butanol yield (0.163 g per g of xylose) (Table 75). By using NH_4OH instead of IR45 to neutralize the hydrolyzates, the fermentation was greatly inhibited, again suggesting the presence of inhibitors in wood hemicellulose hydrolyzates which can be partially removed by IR 45 resin treatment. This would then explain our unsuccessful attempts in the bioconversion of acid hydrolyzed water-solubles neutralized by NH_4OH (Table 74) and the enzymatically hydrolyzed water-solubles as well as the water-insolubles (Table 76). A study on the inhibitors inherent in the substrate and/or enzyme preparation will be required for the acetone-butanol fermentation to operate on natural wood substrates.

Discussion

For the efficient bioconversion of hemicellulose to liquid fuels it is essential that complete utilization of all the major sugars present be achieved. Although Clostridium acetobutylicum can consume all hexoses and pentoses tested, production of acetone and butanol was efficient only with D-glucose, D-mannose and D-cellobiose (Mes-Hartree and Saddler, 1982). Since D-xylose is a major component of hemicellulose (Timell, 1967), the failure of the organism to produce solvents from D-xylose resulted in negligible butanol yield from hemicellulose hydrolyzate (Saddler et al, 1983). It is therefore crucial that improvement in butanol yield from D-xylose be achieved before any bioconversion process utilizing natural hemicellulose substrates can be contemplated.

By using more stringent anaerobic conditions for preparing culture media, together with the use of hydrogen gas in headspace, the addition of a reducing agent (cysteine-hydrochloride) was found to be unnecessary for the maintenance of a proper anaerobic environment for culturing the organism and was actually detrimental to the production of solvents. The exact nature of cysteine inhibition is at present not known. Considering that the organism can grow in a wide range of redox potential (Eh from +370 mV to -400 mV in the absence of oxygen) (O'Brien and Morris, 1971), it is unlikely that the inhibition was due to changes in Eh. A more likely explanation could be the formation of hydrogen peroxide from the oxidation of cysteine during medium preparation. Since Clostridium spp. are known to lack in catalase, the presence of trace quantities of hydrogen peroxide could inhibit growth of the organism and indirectly lead to a decrease in solvent yields. Our findings that cell growth and sugar utilization of Clostridium acetobutylicum decreased with increasing concentrations of cysteine in medium lends support to this hypothesis.

Under the present culture conditions, solvent production by C. acetobutylicum grown on all major sugars present in wood hemicellulose, except D-galactose, has been significantly improved over previous yields (Mes-Hartree and Saddler, 1982). D-glucose, D-mannose, D-cellobiose and L-arabinose could all be efficiently utilized for solvent production with 0.246-0.267 g butanol being produced per g of substrate utilized, or 90.9-98.8% of the theoretical yield (Wang et al, 1977). Butanol yield from D-xylose, now approached 0.156 g/g D-xylose consumed (57.9% of theoretical yield), but the butanol level attained was still relatively low. The low butanol level, however, was noted to be accompanied by an accumulation in butyric acid (Mes-Hartree and Saddler, 1982), suggesting that the normal fermentation process has stopped prior to the phase of solvent production (Davies and Stephenson, 1941). We therefore attempted to induce the acid to solvent conversion by the addition of acetic or butyric acid, an approach recently demonstrated to be successful with glucose-grown cells (Bahl et al, 1982; Gottschal and Morris, 1980; Haggstrom and Molin, 1980).

Under the conditions used in this study, acid addition did not significantly improve solvent production of cells grown on D-glucose With cells grown on D-xylose, on the other hand, a dramatic increase in acetone and butanol was obtained by the addition of either acid, particularly if the addition occurred prior to initiation of fermentation. Earlier workers reported that the addition of acetate to cultures only led to an increase in acetone production with no effect on butanol formation, while butyrate addition resulted in enhanced butanol production (Nakhmaninovich and Sheblikina, 1960). Such an effect was attributed to the conversion of the added acids directly to their respective solvents and appeared to be common in other organisms carrying out acetone-butanol fermentation (Prescott and Dunn, 1954). Our study here clearly shows that the addition of either acetic or butyric acid to D-xylose grown cells resulted in increased yields of both acetone and butanol to an extent which cannot be accounted for solely by the conversion of the relatively low levels of acids added. The finding that acid addition prior to fermentation was more effective in enhancing solvent yields also indicated that pH effect by itself probably is not responsible. Although the mechanism of this stimulation is not defined at present, it appears likely that enzyme induction may be involved. Further refined studies will be useful in determining the roles played by these acids in the solvent production of C. acetobutylicum. This is particularly important for the utilization of the wood hemicellulose, since the steam-treatment led to a release of acetyl groups present in the wood hemicellulose. It may therefore be necessary to partially remove the acetate in the substrate so as to lower its concentration to sub-inhibitory levels, or hopefully stimulatory level.

Our study on the utilization of wood hemicellulose hydrolyzates indicated the presence of inhibitors in the substrates. Although the identity of such inhibitors is at present not determined, it is likely that acetic acid plays a role in the inhibition, particularly at high substrate level. It is nevertheless encouraging that such inhibitors could be readily removed by IR 45 resin to render the substrates utilizable. The final butanol yields of up to 0.163 g/g of wood hemicellulose sugars and 0.263 g/g of wood cellulose sugars strongly suggest that C. acetobutylicum is a good candidate for the bioconversion of biomass to liquid fuels and chemical feedstocks.

General Discussion

Research in this laboratory has demonstrated the technical feasibility for the utilization of wood hemicellulose-derived sugars for the production of valuable fuels and chemicals. There are however some limitations which must be overcome before the industrial application of the process can be contemplated. The major problem at this point appears to be the presence of fermentation inhibitors inherent in the wood hemicellulose hydrolyzates, thereby preventing the possible use of high substrate concentrations required to achieve the desirable product levels for economical recovery. The identity of the inhibitors has not been determined, but could foreseeably be furfural, hydroxymethylfurfural and phenolic derivatives of lignin degradation products. It is therefore crucial to initiate a study for the identification and possible removal of such inhibitors. Alternatively, our success in the approaches of acclimatization and fed-batch with butanediol fermentation suggests that similar attempts may be feasible to circumvent the inhibitor problem, as well.

Another area of concern is in the hydrolysis process required for the release of fermentable sugars from wood hemicellulose. Although enzymatic hydrolysis of steam-treated wood substrates could be carried out at efficiencies comparable to acid hydrolysis, the cost of the enzyme production is at present prohibitive. Moreover, enzyme preparations in the form of concentrated culture filtrates were inhibitory to subsequent fermentation, presumably due to the accompanying high concentrations of fungal metabolites and medium ingredients. These, generally, can be readily removed by partial purification of the enzyme(s) involved but the process is not likely to be economically viable. An alternative approach could be to carry out a combined hydrolysis and fermentation process. This approach will provide the fermenting organism the time period for acclimatizing to the inhibitors present in the substrate and/or enzyme preparation. It will also improve the hydrolysis efficiencies of the enzymes by the continual removal of end-product inhibitors of the enzyme(s), i.e. glucose, cellobiose, xylose and xylobiose by the microorganisms for solvent production. This in turn can lessen the need for more concentrated enzyme levels otherwise required to achieve the same hydrolysis efficiencies. This area of research will be studied in depth in our future program.

By using two nutritionally versatile microbes capable of efficient bioconversion of all the major hexoses and pentoses present in wood, we also demonstrated the feasibility for the utilization of both wood cellulose and hemicellulose by a single process. This is particularly interesting in that the enzyme preparations used contain active cellulase as well as xylanase enzyme activities. It should therefore be possible to carry out the bioconversion of wood by an approach consisting of a single enzyme preparation and a single fermenting organism (or a mixed culture of an enzyme-producing and a fermenting organism). If such a simplified approach to wood utilization was successful it would definitely be valuable for future industrial considerations.

References

Ackman, R.G. 1972. Porous polymer bead packings and formic acid vapour in the GLC of volatile free fatty acids. J. Chromatogr. Sci. 10: 560-565.

Bahl, H., W. Anders, K. Braun and G. Gottschal. 1982. Effect of pH and butyrate concentration on the production of acetone and butanol by Clostridium acetobutylicum grown in continuous culture. Eur. J. Appl. Microbiol. Biotechnol. 14: 17-20.

Bisaria, J.S. and T.K. Ghose. 1981. Biodegradation of cellulosic materials, substrates, microorganisms, enzymes and products. Enzyme Microbial Technol. 3: 90-104.

Davies, R. and M. Stephenson. 1941. Studies on acetone-butyl alcohol fermentation. Biochem. J. 35: 1320-1331.

Esener, A.A., G. Bol, N.W.F. Kossen and J.A. Roels. 1980. Effect of water activity on microbial growth. p. 339-344. IN Advances in Biotechnology. Vol. 1. Pergamon Press, Toronto.

Flickinger, M.C. 1980. Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come? Biotechnol. Bioeng. 22S: 27-48.

Freeman, G.G. 1947. The fermentation of sucrose by Aerobacter aerogenes. Biochem. J. 41: 389-398.

Freeman, G.G. and R.I. Morrison. 1947. Production of 2,3-butylene glycol by fermentation of molasses. J. Soc. Chem. Ind. 66: 216-221.

Gong, C-S, L.F. Chen, M.C. Flickinger and G.T. Tsao. 1982. Conversion of hemicellulose carbohydrates. IN Feichter, A. (Ed.) Advances in Biochem. Engineering. pp. 93-118. Springer-Verlag. Berlin, Heidleberg, N.Y.

Gottschal, J.C. and J.G. Morris. 1981. The induction of acetone and butanol production in cultures of Clostridium acetobutylicum by elevated concentrations of acetate and butyrate. FEMS Microbiol. Lett. 12: 385-389.

Haggstrom, L. and N. Molin. 1980. Calcium alginate immobilized cell of Clostridium acetobutylicum for solvent production. Biotechnol. Lett. 2: 241-246.

Herrick, F.W. and H.L. Hergert. 1977. The structure, biosynthesis, and degradation of wood. Recent Adv. Phytochem. 11: 443-515.

Horwath, J.A., R. Mutharasan and E.D. Grossman. 1983. Pentosan hydrolysis in a concentrated slurry system. Biotechnol. Bioeng. *25*: 19-32.

Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. RW. *14*: 1-29.

Jansen, N.B. 1982. Application of bioenergetic principles to modelling the batch fermentation of D-xylose to 2,3-butanediol by *Klebsiella pneumoniae*. Ph.D. Thesis, Purdue University, W. Lafayette, Indiana.

Jeffries, T.W. 1981. Conversion of xylose to ethanol under aerobic conditions by *Candida tropicalis*. Biotechnol. Lett. *3*: 213-218.

Johansen, L., K. Bryn and F.C. Stormer. 1975. Physiological and biochemical pathway in *Aerobacter* (*Enterobacter*) *aerogenes*. J. Bacteriol. *123*: 1124-1130.

Jones, T.K.N., C.B. Purves and T.E. Timell. 1961. Constitution of a 4-O-methylglucuronoxylan from the wood of trembling aspen (*Populus tremuloides* Michx.). Can. J. Chem. *39*: 1059-1066.

Koshijima, T., T.E. Timell and M. Zimbo. 1965. The number-average molecular weight of native hardwood xylan. J. Polymer Sci. *11*: 265-279.

Ledingham, G.A. and A.C. Neish. 1954. Fermentative production of 2,3-butanediol pp. 27-93. *IN* Underkofler, L.A. and R.J. Hickey (Eds) Industrial fermentations. Vol. 2, Chemical Publishing Co. Inc., N.Y.

Lee, Y.Y., C.M. Lin, T. Johnson and R.P. Chambers. 1979. Selective hydrolysis of hardwood hemicellulose by acids. Biotechnol. Bioeng. Symp. *8*: 75-88.

Lee, Y.Y. and T.A. McCaskey. 1982. Hemicellulose hydrolysis and fermentation of resulting pentoses to ethanol. TAPPI Research and Development Division Conference. pp. 317-325.

Long, S.K. and R. Patrick. 1961. Production of 2,3-butylene glycol from citrus wastes. I. The *Aerobacter aerogenes* fermentation. Appl. Microbiol. *9*: 244-248.

Long, S.K. and R. Patrick. 1963. The present status of the 2,3-butylene glycol fermentation. pp. 135-155. *IN* Umbrient, W.W. (Ed.). Advances in Applied Microbiology. *Vol*. 5. Academic Press, N.Y.

Lowry, O.H., N.R. Rosebrough, A.L. Farr and R.J. Randal. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 93: 265-275.

Maddox, I.S. 1980. Production of n-butanol from whey filtrate using Clostridium acetobutylicum N.C.I.B. 2951. Biotechnol. Lett. 2: 493-498.

Mandels, M., R. Andreotti and C. Roche. 1976. Measurement of saccharifying cellulose. Biotechnol. Bioeng. Symp. 6: 21-34.

McCall, K.B. and C.E. Georgi. 1954. The production of 2,3-butanediol by fermentation of sugar beet molasses. Appl. Microbiol. 2: 355-359.

Mes-Hartree, M. and J.N. Saddler. 1982. Butanol production of Clostridium acetobutylicum grown on sugars found in hemicellulose hydrolyzates. Biotechnol. Lett. 4: 247-252.

Miller, G.L. 1959. Use of dinitrosalicylic reagent for the determination of reducing sugars. Anal. Chem. 31: 426-428.

Miller, T.L. and M.J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. 27: 985-987.

Montenecourt, B.S. and D.E. Eveleigh. 1977. Semi-quantitative plate assay for determination of cellulase production by Trichoderma viride. Appl. Environ. Microbiol. 33: 178-183.

Nakhmanovich, B.M. and N.A. Shcheblykina. 1960. Fermentation of calcium salts of acetic and butyric acids by Clostridium acetobutylicum. Mikrobiol. 29: 67-72.

O'Brien, R.W. and Morris, J.G. 1971. Oxygen and the growth and metabolism of Clostridium acetobutylicum. J. Gen. Microbiol. 68: 307-318.

Olson, B.H. and M.J. Johnson. 1948. The production of 2,3-butylene glycol by Aerobacter aerogenes 199. J. Bacteriol. 55: 209-222.

Perlman, D. 1944. Production of 2,3-butylene glycol from wood hydrolyzates. Ind. Agric. Chem. 36: 803-804.

Pirt, S.J. and D.S. Callow. 1959. Exocellular product formation by microorganisms in continuous culture. II. Production of 2,3-butanediol from sucrose by Aerobacter aerogenes in a two-stage culture. Selected Sci. Papers from the 1st Sup. di Sanita 2: 292-313.

Prescott, S.C. and C.G. Dunn. 1954. The acetone-butanol fermentation. IN Industrial Microbiol. 3rd Ed. McGraw Hill Book Co., N.Y., pp. 250-283.

Reynolds, H., B.J. Jacobson and C.H. Werkman. 1937. The dissimilation of organic acids by Aerobacter indologenes. J. Bacteriol. 34: 15-20.

Rosenberg, S.L. 1980. Fermentation of pentose sugars to ethanol and other neutral products by microorganisms. Enzyme Microbial Technol. 2: 185-193.

Saddler, J.N. 1982. Screening of highly cellulolytic fungi and the action of their cellulase enzyme systems. Enzyme Microbial Technol. 4: 414-418.

Saddler, J.N., H.H. Brownell, L.P. Clermont and N. Levitin. 1982. Enzymatic hydrolysis of cellulose and various pretreated wood fractions. Biotechnol. Bioeng. 24: 1389-1402.

Saddler, J.N., E.K.C. Yu, M. Mes-Hartree, N. Levitin and b.H. Brownell. 1983. Utilization of enzymatically hydrolyzed wood hemicelluloses by microorganisms for the production of liquid fuels. Appl. Enfiron. Microbiol. 45: 153-160.

Schneider, H., P.Y. Wang, Y.K. Chan and R. Maleszka. 1981. Conversion of D-xylose into ethanol by the yeast Pachysolen tannophilus. Biotechnol. Letts. 3: 89-92.

Shields, R. and W. Burnett. 1960. Determination of protein bound carbohydrate in serum by a modified anthrone method. Anal. Chem. 32: 885-887.

Spivey, J.J. 1978. The acetone/butanol/ethanol fermentation. Process Biochem. 11: 2-12.

Stahyl, G.L. and C.H. Werkman. 1942. Origin and relationships of acetylmethylcarbinol to 2,3-butylene glycol in bacterial fermentations. Biochem. J. 36: 575-581.

Timell, T.E. 1967. Recent progress in the chemistry of wood hemicellulose. Wood Sci. Technol. 1: 45-70.

Volesky, B., A. Mulchandain and J. Williams. 1981. Biochemical production of industrial solvents (acetone-butanol-ethanol) from renewable resources. Ann. N.Y. Acad. Sci. 369: 205-218.

Wang, D.I.C., C.L. Cooney, A.L. Demain, R.F. Gomez and A.J. Sinsky. 1979. Degradation of cellulosic biomass and its subsequent utilization for the production of chemical feedstocks. U.S. Dept. of Energy Report No. EG 775024198, Progress Report, Sept. 1 - Nov. 30.

Wang, D.I.C. and H.Y. Fang. 1980. Ethanol production by a thermophilic and anaerobic bacterium using xylose. IN Proc. Am. Chem. Soc. Meet., San Francisco, August. pp. 639-649.

Wentz, F.E., A.D. Marcy and M.J. Gray. 1982. Analysis of wood sugars in pulp and paper industry samples by HPLC. J. Chromatogr. Sci. 20: 349-352.

Wiegel, J., L.G. Ljungdahl and J.R. Rawson. 1979. Isolation from soil and properties of extreme thermophile *Clostridium thermohydrosulfuricum*. J. Bacteriol. 139: 800-810.

Wise, L.E., M. Murphy and A.A. D'Addieco. 1946. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the holocellulose. Paper Trade J. 122: 11-19.

Yu, E.K.C., N. Levitin and J.N. Saddler. 1982. Production of 2,3-butanediol by *Klebsiella pneumoniae* grown on acid hydrolyzed wood hemicellulose. Biotechnol. Lett. 4: 741-746.

Yu, E.K.C. and J.N. Saddler. 1982a. Power solvent production by *Klebsiella pneumoniae* grown on sugars present in wood hemicellulose. Biotechnol. Lett. 4: 121-126.

Yu, E.K.C. and J.N. Saddler. 1982b. Enhanced production of 2,3-butanediol by *Klebsiella pneumoniae* grown in high sugar concentrations in the presence of acetic acid. Appl. Environ. Microbiol. 44: 777-784.

Table 1. Analyses of Products from High Pressure Steaming of Aspen[a]

Temperature	Time (sec)	Yield (% of wood)	Yield of Water Solubles (% of wood)	Pentosan in Water Solubles (% of WS)	Methanol Solubles of WS (% of WS)	Pentosan in MS (% of MS)	Pentosan in WS (% of wood)	Pentosan in Water Insolubles (% of WI)	Pentosan in WI (% of wood)	Pentosan in WS and WI (% of wood)	Lignin in WS (%)	Lignin in WI (%)
240°C	15	96.0	15.6	53.0	26.2	33.7	8.3	11.4	9.0	17.3	16.2	24.0
	30	94.2	18.0	53.8	34.0	48.5	9.7	8.0	6.0	15.7	14.2	23.8
	60	89.0	19.4	54.3	95.7	54.0	10.5	4.0	2.8	13.3		
	90	87.8	20.2	45.5	96.0	45.0	9.2	2.2	1.5	10.7		
	120	85.8	18.4	41.1	95.6	40.2	7.6	1.5	1.0	8.6		
250°C	15	95.2	22.5	51.4			11.6	7.3	5.3	16.9	12.2	25.3

[a] Pentosan of original Aspen = 20.3%

Table 2. Analyses of Products from Acid-Impregnated Aspen Steamed under High Pressure

Temperature	Time (sec)	Additive (% of wood)	Yield of Steamed Wood	Yield of Water Solubles (% of wood)	Pentosan in WS (% of WS)	Methanol Solubles of WS (% of WS)	Pentosan in MS (% of MS)	Pentosan in WS (% of wood)	Pentosan in Water Insolubles (% of WI)	Pentosan in WI (% of wood)	Pentosan in WI and WS (% of wood)	Lignin in WS (%)	Lignin in Water . Insolubles (%)
240°C	15	0.2% H_2SO_4	96.7	21.6	52.6	50.7	40.7	11.4	8.2	6.2	17.6	17.2	24.2
240°C	30	0.2% H_2SO_4	91.9	23.9	51.2	68.6	47.3	12.2	5.2	3.5	15.7		
239°C	15	0.4% H_2SO_4	90.6	27.8	50.6	92.4	51.4	14.1	4.3	2.7	16.8	12.9	26.6
238°C	30	0.4% H_2SO_4	83.8	22.0	53.3			11.7	3.0	1.9	13.6		27.2
239°C	60	0.4% H_2SO_4	78.0		Dark-coloured product - highly degraded								
243°C	180	0.4% H_2SO_4	76.6	16.8	19.0	Dark-coloured product - highly degraded							
240°C	15	1% H_2SO_4	57.6	28.3		Dark-coloured product - highly degraded							52.0
240°C	15	0.2% TFA		23.8	44.4			10.6	7.8	5.9	16.5		

Table 3. Hydrolysis of xylan (Sigma) by dilute H_2SO_4 at 125°C

Acid concentration (%)	Ratio of acid to sample (v/v)	Duration of hydrolysis (h)	% xylose	% theoretical yield
3.0	10:1	1.0	50.1	57.6
5.0	10:1	1.0	51.4	59.1
3.0	5:1	1.5	48.1	55.3
3.0	6:1	1.5	49.7	57.1
3.0	10:1	1.5	48.2	55.4
5.0	10:1	1.5	51.4	59.1
3.0	10:1	2.5	49.9	57.3
5.0	10:1	2.5	43.7	50.2
Pretreated with 72% H_2SO_4 for 2 hr and then diluted to 3.0%		1.0	49.2	56.6

Table 4. Analysis of products from acid hydrolyzed wood substrates[a]

Substrates[b]	Pentosan content (%)	% xylose (based on wt. of sample hydrolyzed)	% theoretical yield
Xylan (Sigma)	76.6	53.8	61.8
Aspen	20.3	46.1	50.6
SEW-WS	51.4	46.7	80.0
SEW-WS-MS	54.0	41.2	67.2
SEW-WS-A	53.0	34.2	56.8
SEW-WS-MS-A	51.4	43.2	74.0

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[a] Acid hydrolysis was carried out with 3% H_2SO_4 (acid to sample ratio, 10:1, v/v) at 125°C for 1h

[b] Xylan and aspen wafers were acid hydrolysis without any pretreatment. All other substrates were steam-exploded and then extracted by water and methanol: SEW-WS, steam-exploded aspen (250°C, 15S) water-solubles; SEW-WS-MS, steam-exploded aspen (240°C, 60S) water and methanol solubles; SEW-WS-A, steam-exploded aspen presoaked in 0.2% H_2SO_4 (240°C, 15S), water solubles; SEW-WS-A-MS, steam-exploded aspen presoaked in 0.4% H_2SO_4 (240°C, 15S) water and methanol solubles.

Table 5. Hydrolysis of aspen xylan by dilute H_2SO_4 at 100°C

Sample	Acid concentration (%)	Substrate concentration (in acid)	Duration of Treatment (h)	Reducing sugars released (g/L)	D-xylose released	% Original sample
Xylan	1.0	5.07 g/100 cc	4	39.5	22.3	44.0
Xylan	3.0	2.50 g/50 cc	2	35.0	32.4	64.8
Xylose (control)	3.0	2.50 g/50 cc	2	44.1	43.6	87.2
Xylan	3.0	5.00 g/100 cc	4	37.9	32.2	64.4
Xylose (control)	3.0	5.05 g/100 cc	4	34.4	34.5	68.3
Xylan	3.0	2.50 g/100 cc (final volume 50 cc)	2	36.8	31.3	62.5
Xylan	3.0	5.04 g/560 cc (final volume 100 cc)	4	45.1	28.1	55.7
Xylose (Control)	3.0	5.03 g/560 cc (final volume 100 cc)	4	51.6	44.8	89.1

Table 6. Product analysis of acid hydrolyzed wood hemicellulose[a]

Samples	Conc'n (g/100 cc acid)	Reducing sugars released (g/L)	Monosaccharides (D-xylose) (g/L)	
Xylan	5	40.81	32.5	(65.0)[b]
	10	80.09	75.9	(75.9)
SEW-WS[c] 15S	5	28.46	17.0	(34.0)
	10	59.60	40.7	(40.7)
SEW-WS 30S	5	29.33	23.4	(46.8)
	10	58.20	45.9	(45.9)
SEW-WS 60S	5	29.16	11.9	(23.8)
	10	58.58	40.0	(40.0)

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[a] Substrates were hydrolyzed with 3% H_2SO_4 at the concentrations stated at 100°C for 4h, neutralized by NH_4OH and reconstituted to 100 ml

[b] % of original sample

[c] SEW-WS: steam exploded aspen, water solubles after various durations of steam treatment (15S, 30S and 60S)

Table 7. Acid hydrolysis of wood hemicellulose substrates[a]

Samples	Total reducing sugars (g/L)	Monosaccharides (HPLC, g/L)			Acetic Acid (5% sol'n) (g/L)
		Glucose	Xylose	Total	
SEW-WS 15S	41.58	1.6	28.4	30.0 (31.9)[b]	1.94
SEW-WS 60S	53.25	4.2	42.0	46.2 (30.2)	1.91
SEW-WS 30S	72.93	3.4	49.8	53.2 (35.9)	1.66

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[a] Substrates were hydrolyzed by 3% H_2SO_4 at 100°C for 4h, neutralized by IR45 resin and concentrated

[b] % of original sample

Table 8. Analysis of sugars released from acid hydrolysis of wood substrates

Samples	Acid Treatment	Neutralization agent	Reducing sugars (g/L)	Glucose (by glucostat)	Sugars (HPLC) (g/L)			
					Glucose	Cellobiose	Xylose	Total
SEW-15S WI	72% +3%	IR45	172.60 (71.1)[b]	122.58 (50.6)	72.7 (29.9)	1.1	12.9	86.7
SEW-30S WI	72% +3%	IR45	144.20 (52.3)	168.82 (61.4)	112.5 (40.9)	3.5	9.5	125.5
SEW-60S WI	72% +3%	IR45	218.35 (97.0)	101.08 (44.9)	84.7 (37.6)	1.9	3.5	90.1
Holocellulose	72% +3%	IR45	159.89 (59.8)	167.70 (62.2)	81.4 (30.5)	1.6 (0.60)	28.4 (10.6)	111.4
Aspen	3%	IR45	34.57 (15.4)	1.02	1.5	ND[c]	20.0 (8.9)	21.5
Holocellulose	3%	NH_4OH	26.16 (16.5)	0.28	0.9	ND	20.4 (12.8)	21.3
Holocellulose	3%	IR45	22.17 (17.4)	0.44	1.0	ND	19.4 (15.2)	20.4

[a] Wood substrates were hydrolyzed by 72% or 3% H_2SO_4 as indicated.
SEW-WI, steam-exploded wood, water insolubles

[b] % of original sample

[c] Not detected

Table 9. Cellulase and xylanase activities of various *Trichoderma* sp grown for 10 days on 2% Solka floc

Trichoderma strain	Extracellular protein (mg/ml)	Filter paper activity (IU/ml)	β-glucosidase activity (IU/ml)	Endoglucanase activity (IU/ml)	Xylanase activity (IU/ml)
D39	1.0	0.8	0.5	20.2	20
C449	1.1	1.2	0.4	41.5	213
C332	0.7	0.2	0.8	0.9	90
D59	1.0	2.0	0.5	11.6	128
C280	0.9	1.0	1.6	28.9	35
C415	1.0	0.8	0.4	10.5	119
C461	1.1	1.4	0.6	21.9	188
E58	1.4	3.1	1.3	147.4	250
C30	2.5	5.6	0.3	312.6	400
QM9414	1.6	3.4	0.2	203.7	372

Table 10. Production of cellulase and xylanase enzymes by *Trichoderma* E58 grown on solka floc

Incubation Time (days)	Protein (mg/ml)	CMCase (IU/ml)	Salicinase (IU/ml)	Filter Paper (IU/ml)	Xylanase (IU/ml)
2	0.77	5.9	0.46	0.26	114.0
4	0.91	10.8	0.44	0.68	458.7
7	1.57	51.5	1.92	1.59	298.7
9	1.40	40.6	1.80	1.91	266.7

Table 11. Cellulase and xylanase production of *Trichoderma reesei* C30 and *T. harzianum* E58 grown on various lignocellulosic substrates for 6 days

Trichoderma strain	Substrate	Concentration (mg/ml)	Filter Paper activity (IU/ml)	β-glucosidase activity (IU/ml)	Endoglucanase activity (IU/ml)	Xylanase activity (IU/ml)
T. harzianum E58	solka floc	10	3.2	1.5	60	345
		30	3.5	1.8	65	385
	SE[a]	10	2.8	1.5	45	280
		30	1.8	1.0	25	110
	SE W[b]	10	3.0	1.6	50	340
		30	3.3	1.8	55	365
	SE WA[c]	10	3.5	1.8	60	360
		30	4.0	2.0	70	380
T. reesei C30	solka floc	10	4.8	0.3	130	40
		30	5.0	0.4	145	45
	SE	10	3.8	0.2	110	340
		30	0.2	0.1	40	30
	SE W	10	4.5	0.3	125	200
		30	4.8	0.4	130	230
	SE WA	10	4.9	0.3	135	100
		30	5.2	0.5	100	120

[a] SE; steam exploded aspen, exposed to steam for 20 sec. at 560 psi (250°C)

[b] SE W; Steam exploded aspen which was water extracted

[c] SE W A; Steam exploded aspen which was water and alkali extracted

Table 12. Effect of different storage conditions on the xylanase activity of culture filtrate of *T. harzianum* E58

Storage conditions (for 30 days)	Protein Conc (mg/ml)	Xylanase activity	
		IU/ml	IU/mg
4°C	0.8	177	214
-10°C	0.8	175	215
rotary evaporated, 4°C	6.7	1339	199
ammonium sulphate ppt, 4°C	6.0	1140	191
original	0.8	173	216

Table 13. Cellulase and xylanase profile of *T. harzianum* E58 culture filtrate and NOVO cellobiase preparation dilute to a protein concentration of 1 mg/ml

Enzyme source	Filter Paper activity (IU/ml)	β-glucosidase activity (IU/ml)	Endoglucanase activity (IU/ml)	Xylanase activity (IU/ml)
NOVO cellobiase	2.1	10.0	0.2	12.0
T. harzianum E58	2.0	0.9	106.5	310.0

Table 14. Hydrolysis of various concentrations of xylan by *T. harzianum* E58 culture filtrate and NOVO cellobiase diluted to a protein concentration of 1 mg/ml

Enzyme source	xylan concentration (mg/ml)	incubation time (min)		
		30	60	120
		reducing sugars (mg/ml)		
T. harzianum E58	20	5.6	6.8	8.7
	50	11.0	12.1	17.2
	100	17.2	19.4	30.6
NOVO cellobiase	20	8.0	9.4	14.9
	50	14.1	18.5	34.2
	100	25.6	27.7	110.7

Table 15. Comparison of xylanase enzyme assay using various xylan preparations as substrates[a]

Substrate	Source	Xylanase activities IU/ml	 IU/mg
Xylan (Larchwood)	Sigma	259	386
Xylan (Oat Spelts)	Sigma	479	715
Xylan (Aspen)	Forintek	337	502

[a] Enzyme preparation was obtained from culture filtrate of *T*. E58 grown on solka floc.

Table 16. Enzyme activities of *Trichoderma* E58 culture filtrate preparations

Enzyme preparation	Protein (mg/ml)	CMC ase		Salicinase		Filter Paper		Xylanase	
		IU/ml	IU/mg	IU/ml	IU/mg	IU/ml	IU/mg	IU/ml	IU/mg
Original filtrate	1.01	22.5	22.3	1.20	1.19	1.04	1.03	529.1	523.8
Filtrate conc[d] by Rotary evaporation	7.30	121.5	16.7	2.90	0.40	6.15	0.84	1365.0	187.0

Table 17. Enzymatic hydrolysis of wood substrates by Trichoderma E58 filtrates[a]

Substrate	Substrate conc^n (%)	Reducing sugars released (g/L)	Monosaccharides (g/L)			Acetic Acid (g/L)
			glucose	xylose	total	
Holocellulose	5	11.42	5.7	2.0	7.7	1.60
	10	14.09	5.3	3.0	8.3	1.83
SEW-WI-15S	5	5.80	1.4	1.7	3.1	1.31
	10	10.86	2.9	3.8	6.7	2.39
SEW-WI-30S	5	1.93	0.0	0.0	0.0	0.53
	10	8.02	1.7	1.2	2.9	1.17
SEW-WI-60S	5	1.65	0.0	0.0	0.0	0.28
	10	4.22	1.2	0.4	1.6	0.56
Xylan	5	24.56	0.0	8.7	8.7	1.07
	10	44.00	0.0	16.8	16.8	1.97
SEW-WS-15S	10	30.22	2.6	13.4	16.0	7.80
SEW-WS-30S	10	41.37	2.3	20.0	22.3	13.02
SEW-WS-60S	10	48.60	3.7	20.5	24.2	16.25

[a] Hydrolysis was carried out using culture filtrate concentrated by rotary evaporation (Table 16) at 50°C for 1h.

Table 18. Xylanase enzyme activities of *T.* E58 filtrates

Enzyme Preparation	Xylanase activities		β-xylobiase activities	
	IU/ml	IU/mg	IU/ml	IU/mg
Original filtrate	304.76	516.54	0.47	0.80
Conc^d filtrate (Rotary evaporation)	2081.47	473.06	3.79	0.86
Conc^d filtrate (Pelicon membrane filtration)	1612.61	359.96	4.36	0.97

Table 19. Comparison of enzymatic hydrolysis of 5% xylan under various conditions

Enzyme preparations	Enzyme Conc'n (%)	Reducing sugars released (g/L)					Sugars (HPLC)					
		pH 5.0, 50°C		pH 6.5, 30°C			pH 5.0, 50°C				pH 6.5, 30°C	
		1d	2d	1d	2d	3d	1d		2d		1d	
							Xylose	Xylobiose	Xylose	Xylobiose	Xylose	Xylobiose
A) Original filtrate	5%	12.2	13.1	10.8	12.8	13.0	1.8	3.8	2.3	3.9	ND	5.5
	10%	12.7	14.4	11.2	12.8	14.1	2.3	4.4	3.5	5.9	ND	4.7
B) Conc^{d} by Rotary evaporation	5%	17.1	20.1	14.4	17.4	18.6	8.5	4.6	10.6	11.3	3.5	7.3
	10%	18.5	19.7	15.4	18.0	20.6	10.2	4.8	12.2	3.6	5.7	7.6
C) Conc^{d} by Pelicon membrane filtration	5%	17.4	19.2	14.5	16.9	17.4	7.8	4.9	9.1	4.4	4.0	7.4
	10%	17.9	20.2	15.5	18.5	19.0	11.1	4.0	13.6	3.2	5.7	7.4

Table 20. Enzymatic hydrolysis of xylan using various preparations of *T*. E8 culture filtrates

Xylan conc'n (g/L)	Enzyme preparation	Enzyme conc'n (%)	Hydrolysis Time (d)	Reducing sugars released (g/L)
50	A) Original filtrate	5	1	16.4
			3	21.4
		10	1	20.0
			3	21.0
	B) Conc 7X (Rotary evap.	5	1	14.5
			3	21.3
		10	1	24.8
			3	24.8
	C) Conc 15X (Pelicon memb.)	5	1	21.6
			3	21.8
		10	1	28.1
			3	28.5
100	A) Original filtrate	5	1	27.0
			3	28.3
		10	1	36.5
			3	39.2
	B) Conc 7X (Rotary evap.)	5	1	24.1
			3	24.5
		10	1	48.0
			3	48.2
	C) Conc 15X (Pelicon memb.)	5	1	24.8
			3	36.9
		10	1	49.4
			3	49.5

Table 21. Product analysis of enzymatically hydrolyzed wood hemicellulose[a]

Substrate	Concentration (g/L)	Reducing sugars released (g/L)[b]	Reducing sugars released (g/L)[c]		
			Day 2	Day 5	Day 10
Xylan	20	9.27	8.85	10.34	10.95
	50	22.68	23.20	22.80	30.30
	100	50.48	47.20	49.80	67.80
SEW-WS-15S	20	8.92	9.20	9.16	10.90
	50	31.50	27.30	23.50	26.60
	100	39.91	53.60	48.40	61.40
SEW-WS-30S	20	9.43	8.85	9.98	9.52
	50	20.45	26.60	26.00	26.40
	100	40.31	59.60	60.00	58.00
SEW-WS-60S	20	9.52	7.75	10.46	10.68
	50	18.96	26.20	24.20	29.30
	100	46.81	59.60	54.80	59.40

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[a] Substrates were hydrolyzed by culture filtrate of T. E58 concentrated by Pellicon membrane filtration at final protein concentration of 1.0 mg/ml

[b] Hydrolysis was carried out at 50°C, pH 5.0 for 2 days

[c] Hydrolysis was carried out at 30°C, pH 6.5

Table 22. Activities of various enzymes after membrane filtration

Enzyme preparation	CMC ase		Salicinase		Filter Paper		Xylanase		Protein
	IU/ml	IU/mg	IU/ml	IU/mg	IU/ml	IU/mg	IU/ml	IU/mg	(mg/ml)
Eluant	4.30	11.57	0.09	0.23	0.17	0.46	363.7	983.03	0.37
Conc^{d} filtrate (75X)	2524.9	41.73	107.2	1.77	112.8	1.86	12210.0	201.8	60.50
Eluant	3.06	8.74	0.07	0.21	0.16	0.47	252.9	722.6	0.35
Conc^{d} filtrate (75X) (8 days storage)	1983.20	35.41	94.42	1.69	102.67	1.83	9874.6	176.3	56.00
Conc^{d} filtrate (75X) (14 d storage)	1323.12	28.15	83.47	1.78	72.02	1.53	8418.24	179.11	47.00
Rediluted 5X	333.00	33.00	17.32	1.73	18.45	1.85	1909.20	190.92	10.00

Table 23. Product analysis of wood hemicellulose hydrolyzed by *T. harzianum* E58 enzymes[a]

Substrate	Concentration (g/L)	Reducing sugars released (g/L)	Sugars released (HPLC) (g/L)				
			Glucose	Xylose	Xylobiose	Cellobiose[c]	Total
Xylan	50	34.30	ND[b]	13.5	4.8	ND	18.3
	100	66.80	ND	21.7	10.0	2.7	34.4
SEW-WS-15S	50	33.10	1.0	15.9	0.6	ND	17.5
	100	72.80	1.4	16.2	9.4	4.3	31.3
SEW-WS-30S	50	38.80	ND	18.0	1.3	ND	19.3
	100	74.60	1.1	13.7	11.6	6.5	32.9
SEW-WS-60S	50	36.40	0.7	12.7	3.0	ND	16.4
	100	86.20	ND	14.6	14.2	6.4	35.2

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[a] Substrates were hydrolyzed by *T.* E58 culture filtrates concentrated by Pelicon membrane filtration (without storage). Final protein concentration in hydrolysis reaction mixture was 5 mg/ml. Hydrolysis was carried out at 50°C, pH 5.0 for 1 day.

[b] Not detected

[c] Peaks were detected with RS (retention time factor) corresponding to cellobiose, but could possibly be oligomers of xylose sugars.

Table 24. Hydrolysis of wood hemicellulose by _T._ _harzianum_ E58 culture filtrates[a]

Substrate	Substrate Concentration (g/L)	Reducing sugars released (g/L)
Xylan	20	9.05
	40	13.93
SEW-WS-15S	20	10.40
SEW-WS-30S	20	11.87
	40	25.71
SEW-WS-60S	20	12.86
	40	22.33

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[a] Hydrolysis was carried out with rediluted filtrate concentrated by membrane filtration (Table 22) at $30^{o}C$, pH 6.5 for 2 days

Table 25. Hydrolysis of wood cellulose by _T._ _harzianum_ E58 culture filtrates[a]

Substrate	Concentration (g/L)	Reducing sugars released (g/L)		Sugars released (HPLC) g/L[b]		
		50°C, pH 5.0	30°C, pH 6.5	Glucose	Xylose	Total
Solka floc	50	24.50	19.25	20.6	2.6	23.2
	100	40.01	39.20	30.4	4.1	34.5
SEW-WI-15S	50	9.58	4.30	5.0	2.1	7.1
	100	15.06	7.33	7.0	2.9	9.9
SEW-WI-30S	50	8.80	2.52	4.8	ND[c]	4.8
	100	11.69	3.81	6.4	ND	6.4
SEW-WI-60S	50	10.53	3.44	7.5	ND	7.5
	100	10.52	7.50	6.7	ND	6.7

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[a] Hydrolysis was carried out with _Trichoderma_ E58 culture filtrate concentrated by membrane filtration (Table 22)

[b] Sugars released from hydrolysis of cellulose at 50°C, pH 5.0

[c] Not detected

Table 26. Composition of Kp2 medium:

Ingredients	/L
$K_2HPO_4.3H_2O$	11.4 g
KH_2PO_4	1.5 g
$(NH_4)_2SO_4$	3.0 g
MES	10.0 g
Glacial acetic acid	5.0 g
EDTA	400 mg
$MgSO_4.4H_2O$	240 mg
NaCl	100 mg
$CaCl_2.2H_2O$	14 mg
$FeSO_4.7H_2O$	10 mg
$MnSO_4.4H_2O$	2.8 mg
$ZnSO_4.7H_2O$	7.5 mg
Carbon Source	10.0 - 200.0 g

Table 27. Composition of PA medium

Components	Concentration (g/L)
Xylose	5-150
$K_2HPO_4.3H_2O$	13.7
KH_2PO_4	2.0
$(NH_4)_2HPO_4$	3.3
$(NH_4)_2SO_4$	6.6
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	0.05
$ZnSO_4.7H_2O$	0.001
$MnSO_4.H_2O$	0.001
$CaCl_2.2H_2O$	0.01
EDTA	0.05

Table 28. The effect of sugar preparation on butanediol fermentation of K. pneumoniae[a]

Substrate	Substrate concentration	Sterilization method	Incubation Time (days)	O.D. (540 nm)	% C utilized	Solvents (g/L)			
						Ethanol	Acetic Acid	Butanediol	Acetoin
Glucose	5	Autoclaved	2	5.18	95.0	5.88	0.37	20.64	0.06
			5	6.59	97.8	6.80	0.36	19.24	0.44
			7	5.77	98.6	6.00	0.37	25.15	0.10
		Filtered	2	5.09	97.0	5.92	0.27	20.18	0.06
			5	6.36	98.4	6.48	0.42	19.12	0.40
			7	6.57	99.4	5.77	0.38	24.58	0.14
	10	Autoclaved	2	4.90	59.5	5.97	0.03	24.25	0.05
			5	5.91	59.5	6.44	0.08	28.14	0.12
			7	4.39	60.0	5.65	0.08	26.05	0.08
		Filtered	2	4.95	55.0	5.44	0.17	20.34	0.30
			5	5.44	55.0	7.00	0.04	18.85	0.48
			7	5.41	55.0	5.85	0.09	26.94	0.10
Xylose	5	Autoclaved	2	4.40	41.0	5.47	0.33	18.30	0.08
			5	5.01	79.0	6.31	0.17	26.19	0.10
			7	5.06	94.2	6.12	0.09	25.40	0.07
		Filtered	2	4.49	76.4	5.57	0.30	19.02	0.25
			5	4.59	94.4	6.17	0.21	25.08	0.12
			7	5.21	98.0	5.91	0.13	25.27	0.06
	10	Autoclaved	2	3.53	49.0	2.48	2.10	9.57	0.06
			5	4.00	65.0	5.80	0.10	24.98	0.12
			7	3.91	68.0	5.67	0.10	25.98	0.07
		Filtered	2	3.72	49.0	2.89	1.66	7.61	0.41
			5	4.26	70.0	5.34	0.08	21.57	0.09
			7	3.96	70.0	5.38	0.09	25.22	0.06

[a] Cultures were grown under finite air conditions in Kp2 medium (pH 6.5) and were incubated at 30°C with shaking (150 rpm) for 2-7 days.

Table 29. The production of butanediol by *K. pneumoniae* grown on sugars present in wood hemicellulose hydrolyzates[a]

Substrates	Initial Concentration (%)	O.D. (540 nm)	% sugar utilized	Solvents (g/L)		
				Ethanol	Acetic Acid	2,3-Butanediol
D-Glucose	5	6.25	100.0	4.77	0.06	25.44
	10	2.97	79.0	9.15	0.22	34.65
D-Xylose	5	5.71	98.9	5.97	0.10	27.11
	10	3.77	65.0	5.29	0.16	23.80
D-Galactose	5	4.88	96.2	5.06	0.07	25.41
	10	3.21	56.5	4.88	0.09	26.16
D-Mannose	5	5.88	98.0	5.05	0.04	25.78
	10	4.77	51.5	4.65	0.08	25.29
L-Arabinose	5	5.28	96.8	6.68	0.27	26.00
	10	4.59	77.2	6.50	0.10	21.76
D-Cellobiose	5	3.85	96.0	4.66	0.05	18.01
	10	3.51	46.0	4.58	0.06	16.74

[a] Cultures were grown in Kp2 medium (pH 6.5) under finite air conditions with sugars prepared separately and filter-sterilized. Incubations were carried out at 30°C with shaking (150 rpm) for up to 7 days.

Table 30. Effect of nitrogen supplement on butanediol production of *K. pneumoniae* grown under finite air conditions[a]

Substrate	Substrate conc'n (g/L)	Nutrients added	Sugar utilized(%)	Solvents Ethanol	 Acetic Acid	 Butanediol
D-Glucose	50	None	94.2	5.15	0.11	18.19
		1% YE	99.3	5.07	0.43	20.62
		0.5% urea	99.3	5.40	0.14	24.30
		0.5%$(NH_4)_2SO_4$	99.3	5.49	0.18	23.83
	100	None	47.9	5.07	0.09	22.77
		1% YE	67.4	5.23	0.16	27.70
		0.5% urea	45.4	4.89	0.10	23.90
		0.5%$(NH_4)_2SO_4$	41.9	5.35	0.10	23.35
D-xylose	50	None	91.2	5.13	0.09	21.34
		1% YE	94.0	5.32	0.18	22.28
		0.5% urea	94.1	5.28	1.15	23.90
		0.5%$(NH_4)_2SO_4$	87.7	5.18	0.10	21.80
	100	None	15.1	1.90	1.56	9.81
		1% YE	57.6	5.51	0.14	26.19
		0.5% urea	48.2	5.05	0.10	24.08
		0.5%$(NH_4)_2SO_4$	23.5	0.71	2.54	7.87

[a] Cultures were grown in Ko2 medium and incubated at 30°C with shaking (150 rpm) for 2-7 days for maximal butanediol production

Table 31. Acclimatization of _K. pneumoniae_ to high xylose concentrations under finite air conditions[a]

No. of subcultures	Xylose conc'n (%)	O.D. (540 nm)	Sugar utilization (%)	Solvents produced (g/L) Ethanol	Acetic Acid	Butanediol	Acetoin
0	5	1.90	16.8	0.34	4.52	1.89	ND[b]
1	5	3.06	96.5	6.38	0.06	25.70	ND
2	5	1.78	15.8	0.24	4.51	0.41	0.39
3	5	4.65	80.7	4.43	0.03	17.66	0.43
	10	3.96	17.2	1.95	1.62	9.27	0.57
4	5	4.82	94.5	6.62	0.28	23.79	1.63
	10	4.06	40.0	3.58	1.45	13.16	1.26
5	10	0.94	0.7	0.25	5.37	0.74	0.88
	15	0.10	0.0	0.25	5.17	0.35	0.61
6	10	0.36	0.0	0.12	5.04	1.77	ND
	15	0.05	0.0	0.10	4.48	0.69	ND
7	10	0.075	0.0	0.12	4.37	0.45	ND
	15	0.031	0.0	0.11	5.05	0.33	ND
8	10	0.024	0.0	0.42	5.51	3.34	ND
	15	0.022	0.0	0.33	4.44	1.31	ND
9	10	0.021	0.0	0.09	3.91	1.13	ND
	15	0.034	0.0	0.35	5.04	0.56	0.08

[a] Cultures were grown in Kp2 medium under finite air conditions at 30°C with shaking (150 rpm). Serial subculturing was carried out every 2-3 days using a 2% (v/v) inoculum.

[b] Not Detected

Table 32. Production of butanediol by *K. pneumoniae* under aerobic conditions[a]

Medium[b]	C Source	Sugar concn (g/L)	O.D. 540 nm	% C utilized	Solvents (g/L) Ethanol	Acetic Acid	Butanediol	Acetoin
Kp 2	Glucose	50	7.76	88.6	3.94	0.34	17.08	1.49
		100	8.84	90.5	3.61	0.70	29.78	8.71
	Xylose	50	8.19	95.2	3.18	0.71	19.32	1.84
		100	0.57	17.2	0.19	4.80	6.59	0.24
Kp 1	Glucose	50	11.81	95.1	1.56	0.19	18.16	2.07
		100	9.32	92.4	1.90	1.14	32.67	5.29
	Xylose	50	9.31	94.9	1.54	0.07	17.43	0.58
		100	9.45	67.5	2.37	1.18	21.84	5.21
Kp	Glucose	50	6.04	92.8	1.92	0.29	13.28	3.50
		100	8.92	82.7	2.21	1.08	30.27	4.43
	Xylose	50	10.16	89.0	1.66	0.05	11.04	4.94
		100	10.14	52.4	2.21	0.05	18.85	1.40

[a] Cultures were grown in shake-flasks at 30°C with shaking (150 rpm) for 1-5 days for maximal butanediol production.

[b] Medium Kp 1 is equivalent to Kp 2 except for the deletion of acetic acid. Medium Kp is Kp 2 with both acetic acid and MES removed.

Table 33. Effect of nitrogen supplement on butanediol production by *K. pneumoniae* grown in Kp2 medium aerobically[a]

Substrate	Substrate conc'n (g/L)	Nutrients added[b]	Sugar utilized(%)	Solvents			
				Ethanol	Acetic Acid	Butanediol	Acetoin
D-Glucose	50	None	85.8	2.26	1.70	5.90	6.29
		YE	97.8	3.44	1.58	14.75	0.75
		urea	95.2	3.14	0.81	12.30	1.89
		$(NH_4)_2SO_4$	54.4	2.09	1.61	7.72	0.37
	100	None	81.6	2.46	0.72	20.70	6.04
		YE	84.8	4.95	0.16	28.80	0.24
		urea	92.7	2.94	0.95	21.57	6.11
		$(NH_4)_2SO_4$	95.6	3.37	0.19	28.13	3.29
D-Xylose	50	None	87.6	2.50	1.15	18.74	5.72
		YE	93.3	2.76	1.31	11.62	2.43
		urea	85.1	2.00	1.58	4.98	6.25
		$(NH_4)_2SO_4$	89.7	2.50	1.15	11.13	4.34
	100	None	0.0	0.50	3.71	0.15	0.21
		YE	58.9	4.73	0.14	23.48	0.21
		urea	94.1	2.41	1.09	22.41	5.52
		$(NH_4)_2SO_4$	11.9	0.70	2.72	3.97	0.38

[a] Cultures were grown in Kp2 medium at 30°C with shaking for 1-5 days for maximal butanediol production

[b] Final levels of supplement: yeast extract (YE), 1.0%; urea, 0.5%; $(NH_4)_2SO_4$, 0.5%.

Table 34. Effect of nitrogen supplements on butanediol production of *K. pneumoniae* grown aerobically in Kpl medium[a]

Substrate	Substrate conc'n (g/L)	Nutrients added[b]	Sugar utilized(%)	Solvents Ethanol	Solvents Acetic Acid	Solvents Butanediol	Solvents Acetoin
D-Glucose	50	None	76.2	1.72	0.64	13.28	0.51
		YE	93.3	2.67	ND[c]	16.68	0.31
		urea	90.3	1.31	0.33	11.46	4.87
		$(NH_4)_2SO_4$	84.5	1.52	0.28	12.80	3.95
	100	None	80.4	1.61	0.81	21.05	3.99
		YE	90.1	1.98	0.85	22.71	7.13
		urea	64.3	1.94	ND	23.68	0.30
		$(NH_4)_2SO_4$	77.7	1.51	0.98	24.60	3.69
D-Xylose	50	None	93.8	2.02	0.38	14.86	3.54
		YE	81.1	2.45	0.05	12.58	0.62
		urea	75.0	2.00	0.05	13.12	0.26
		$(NH_4)_2SO_4$	94.4	1.86	0.03	15.81	0.49
	100	None	62.7	1.83	1.33	16.29	3.58
		YE	79.8	2.15	1.38	17.51	5.30
		urea	65.8	1.74	1.22	16.57	3.30
		$(NH_4)_2SO_4$	59.0	1.67	0.92	18.34	3.01

[a] Cultures were grown in Kpl medium (i.e. no HAc) at 30°C with shaking for 1-5 days for maximal butanediol production

[b] Final concentrations of supplements: yeast extract, 1.0%; urea, 0.5% and $(NH_4)_2SO_4$, 0.5%.

[c] Not Detected

Table 35. Acclimatization of *K. pneumoniae* cells in Kpl medium under aerobic conditions[a]

No. of Subculture	Xylose conc'n (%)		Incubation Time (d)	O.D. (540 nm)	% C utilization	Solvents (g/L)			
	in Inoculum	in Medium				Ethanol	Acetic Acid	Butanediol	Acetoin
0	1	5	1	7.20	57.4	1.66	0.34	5.31	0.35
			2	7.65	91.9	2.52	0.70	13.93	3.54
			6	10.80	100.0	1.19	2.73	6.79	6.65
	1	10	1	6.30	2.5	0.85	0.71	2.44	0.27
			2	7.65	42.5	3.34	0.16	19.50	0.83
			6	12.50	89.3	2.42	1.72	23.76	9.28
	1	15	1	4.35	0.0	0.35	0.90	0.59	0.05
			2	6.85	17.5	2.58	0.11	13.72	0.67
			6	12.77	77.2	3.28	1.82	44.25	6.09
1	5	10	1	7.13	26.8	2.72	0.21	12.65	0.21
			5	11.71	93.3	3.40	2.06	28.03	7.04
	5	15	1	6.80	9.2	2.10	0.39	9.70	0.33
			5	12.00	95.6	2.69	2.17	42.31	5.16
2	10	15	4	11.11	94.5	4.78	0.60	45.91	2.54
	15	15	4	10.99	66.6	5.30	0.09	49.04	2.24

[a] Cultures were grown in Kpl medium (i.e. no acetic acid) and were incubated under aerobic conditions at 30^{o}C with shaking (150 rpm). Daily subculturing was carried out, using 2% (v/v) inoculum.

Table 36. Acclimatization of K. pneumoniae cells in PA medium under aerobic conditions[a]

No. of Subculture	Xylose conc'n (%)		Incubation Time (d)	O.D. (540 nm)	% C utilization	Solvents (g/L)			
	in Inoculum	in Medium				Ethanol	Acetic Acid	Butanediol	Acetoin
0	1	5	1	5.75	27.8	0.46	0.74	0.68	0.03
			2	7.92	77.7	2.16	0.33	21.50	2.44
			6	9.60	86.3	1.45	2.30	5.70	6.93
	1	10	1	ND	ND	ND	ND	ND	ND
			2	4.15	0.0	0.36	0.70	0.95	0.03
			6	12.26	91.1	2.65	2.03	23.60	8.86
	1	15	1	ND	ND	ND	ND	ND	ND
			2	0.05	0.0	0.06	0.17	0.24	0.02
			6	12.30	96.0	3.97	0.33	37.28	1.61
1	5	10	1	5.30	4.26	1.35	1.13	3.33	0.21
			5	13.10	91.8	2.31	2.06	27.27	8.84
	5	15	1	1.82	0.0	0.94	0.12	ND	ND
			5	12.60	84.0	3.59	0.99	56.11	2.47
2	10	15	4	12.15	71.3	3.86	0.50	46.33	1.59
	15	15	4	11.11	60.2	4.15	0.89	43.13	1.52

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[a] Cultures were grown in PA medium under aerobic conditions and were incubated at 30^{o}C with shaking (150 rpm). Subculturing was carried out daily using a 2% (v/v) inoculum size.

Table 37. Acclimatization of _K. pneumoniae_ cells grown in Kp2 medium (+ yeast extract) under aerobic conditions[a]

No. of Subculture	Xylose conc'n (%)		Incubation Time (d)	O.D. (540 nm)	% C utilization[b]	Solvents (g/L)			
	in Inoculum	in Medium				Ethanol	Acetic Acid	Butanediol	Acetoin
1	1	5	2	2.45	93.3	1.77	0.80	8.30	1.79
2	5	5	2	4.20	91.6	1.52	0.92	7.30	1.70
		10	2	4.17	79.3	4.14	0.17	21.76	0.24
			5	12.20	89.3	3.51	3.03	31.12	11.64
			7	20.80	87.6	2.55	6.39	33.88	18.5
		15	2	3.97	19.9	3.65	0.04	20.12	0.41
			5	11.78	71.7	5.39	3.85	57.51	7.47
			7	12.85	69.2	3.87	6.55	58.43	12.28
3	5	10	2	10.33	75.4	3.75	0.28	19.27	ND
			5	13.43	90.2	2.90	2.22	29.47	11.87
			7	14.63	86.5	2.29	5.54	28.95	17.36
		15	2	9.14	8.4	3.76	0.18	19.30	0.24
			5	13.24	75.8	4.74	2.95	57.58	8.04
			7	14.50	72.6	3.67	7.23	59.21	13.25
	10	10	2	10.46	76.4	3.85	0.18	20.81	ND
			5	13.45	88.8	3.44	2.32	34.88	11.19
			7	14.24	86.9	2.43	5.85	35.14	15.81

Table 37. (cont'd)

No. of Subculture	Xylose conc'n (%)		Incubation Time (d)	O.D. (540 nm)	% C utilization	Solvents (g/L)			
	in Inoculum	in Medium				Ethanol	Acetic Acid	Butanediol	Acetoin
		15	2	9.00	0.0	3.30	0.04	17.88	0.32
			5	13.20	55.1	4.10	2.52	60.57	7.48
			7	14.44	52.7	ND	7.01	60.35	10.44
	15	15	2	8.93	0.0	3.49	0.15	18.87	0.37
			5	13.50	52.9	4.18	2.53	60.90	7.63
			7	15.20	52.9	1.92	7.77	63.28	10.47

[a] Cultures were grown in Kp2 medium with added yeast extract (1.0%) and were incubated aerobically at 30°C with shaking (150 rpm). Subculturing was carried out at 24h interval using 2% inoculum size

[b] Sugar utilization was based on DNS analysis of reducing sugars remaining in the fermentation medium

Table 38. Effect of acclimatization on butanediol production by K. pneumoniae grown on high xylose concentrations[a]

Culture conditions	Inoculum Preparation[b]	O.D. (540 nm)	% C utilized	Solvents (g/L)			
				Ethanol	Acetic Acid	Butanediol	Acetoin
Finite Air	A	5.77	15.5	7.40	0.36	29.74	1.49
	B	4.59	8.6	7.62	0.25	31.06	1.14
	C	4.66	8.0	7.90	0.23	34.24	1.36
	D	5.24	6.5	ND	0.21	28.17	0.69
	E	3.64	2.3	7.66	0.05	29.67	0.68
Aerobic	A	12.58	55.5	6.03	5.38	53.96	8.69
	B	13.64	54.7	5.37	5.86	59.32	8.90
	C	14.29	55.2	5.44	5.80	62.77	9.76
	D	13.87	53.8	7.12	6.03	68.14	8.33
	E	13.67	52.3	6.02	5.90	67.04	9.01
Aerobic + N_3^- (0.1 - 0.3 g/L)	A	0.36	n/a[c]	0.23	0.57	1.03	ND
	B	0.58	n/a	0.20	0.46	0.38	ND
	C	0.52	n/a	0.22	0.62	0.50	ND
	D	0.34	n/a	0.19	0.50	0.23	ND
	E	0.27	n/a	0.19	0.44	0.21	ND

[a] Cultures were grown in Kp 2 medium (+ 1% yeast extract) (pH 6.5) with 150 g/L of D-xylose. Incubation was carried out at 30°C with shaking (150 rpm) for 2-7 days until maximal butanediol production took place.

[b] Inoculation preparations were obtained through daily subculturing in medium with step-wise increase in xylose levels:
A) 5% - 5% - 10%
B) 5% - 5% - 15%
C) 5% - 10% - 10%
D) 5% - 10% - 15%
E) 5% - 15% - 15%

[c] Not applicable

Table 39. Effect of acclimatization on butanediol production by K. pneumoniae grown in Jansen's PA medium[a]

Culture conditions	Inoculum preparation[b]	O.D. (540 nm)	% C utilized	Solvents (g/L) Ethanol	Acetic Acid	Butanediol	Acetoin
Finite air	A	5.23	6.7	1.55	2.18	10.99	ND
	B	3.69	ND[c]	1.41	1.44	8.45	ND
	C	3.21	2.8	1.03	1.21	5.50	ND
	D	4.04	5.6	1.36	1.18	7.03	ND
	E	3.67	ND	1.07	1.00	5.29	ND
Aerobic	A	15.28	ND	2.39	ND	43.23	0.55
	B	13.40	44.8	1.23	1.26	38.76	1.32
	C	7.61	ND	0.94	1.23	21.69	0.38
	D	13.89	38.1	1.60	ND	59.23	1.74
	E	12.45	57.6	1.54	ND	67.37	2.25

[a] Cultures were grown in Jansen's PA medium (pH 7.1) and incubated at 30°C with shaking (150 rpm) for 7 days

[b] Inoculations were carried out with cells acclimatized according to schemes as of Table 36, except that medium used was PA medium and not Kp 2 medium

[c] Not detected

Table 40. Acclimatization of *K. pneumoniae* to xylan[a]

No. of subculture	Substrate	Solvents (g/L)			
		Ethanol	Acetic Acid	Butanediol	Acetoin
1	Xylan	0.18	3.15	1.54	1.19
	Xylan + glucose	0.20	3.07	1.02	1.21
	Xylan + Xylose	0.32	4.39	1.30	0.41
2	Xylan	0.18	5.04	0.34	0.03
	Xylan + glucose	0.45	5.32	0.98	0.03
	Xylan + Xylose	0.52	4.94	1.18	0.03
3	Xylan	0.13	1.83	0.21	ND
	Xylan + glucose	0.03	1.37	0.01	ND
	Xylan + xylose	0.23	1.76	0.18	ND
4	Xylan	ND[b]	3.29	0.17	0.01
	Xylan + glucose	0.38	4.92	0.81	ND
	Xylan + xylose	0.72	4.89	0.39	ND
5	Xylan	0.13	3.61	0.65	ND
	Xylan + glucose	0.36	4.86	1.04	0.01
	Xylan + xylose	0.63	5.39	0.62	ND
6	Xylan	0.06	3.09	0.36	0.02
	Xylan + glucose	0.71	3.06	0.75	ND
	Xylan + xylose	0.65	1.93	0.94	0.03

[a] Cultures were grown in Kp 2 medium under finite air conditions at 30°C with shaking (150 rpm). Subculturing was carried out weekly using 2% (v/v) inoculum

[b] Not detected

Table 41. Acclimatization of *K. pneumoniae* to uronic acids[a]

Substrate (2%)	No. of subculture	Solvents (g/L)			
		Ethanol	Acetic Acid	Butanediol	Acetoin
Glucuronic acid	0	ND	3.10	1.72	0.91
	1	0.27	5.01	2.93	0.09
	2	0.29	6.75	1.86	0.31
	3	0.31	7.39	2.47	ND[b]
	4	0.40	4.03	2.33	0.03
	5	1.65	7.91	4.22	ND
	6	0.42	7.10	3.37	0.10
Galacturonic acid	0	0.19	3.08	1.36	1.24
	1	0.25	5.44	2.45	0.04
	2	0.48	5.75	1.45	5.28
	3	0.25	7.13	2.48	0.02
	4	0.39	6.88	4.15	0.07
	5	1.57	7.88	1.56	ND
	6	0.47	7.02	1.15	0.06

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[a] Cultures were grown in Kp2 medium under finite air conditions at 30°C with shaking (150 rpm). Subculturing was carried out on a weekly basis

[b] Not detected

Table 42. Butanediol production of *K. pneumoniae* grown on wood hemicellulose hydrolyzates[a]

Samples[b]	Initial reducing sugar conc (g/L)	Initial monosaccharide conc (g/L)	Monosaccharide utilized (%)	Solvents produced (g/L)		
				Ethanol	Acetic Acid	Butanediol
Xylan (Sigma)	12.6	9.3	96.8	1.49	2.26	2.02 (0.22)
	25.1	18.6	97.3	3.00	0.26	8.21 (0.45)
	44.0	32.4	43.5	2.80	0.50	8.32 (0.59)
Aspen	11.2	8.4	94.0	1.06	3.83	3.20 (0.41)
SEW-WS	7.9	6.1	100.0	1.99	2.34	1.66 (0.27)
	15.8	12.1	95.0	3.81	2.14	6.02 (0.52)
	27.6	21.2	13.2	4.20	3.65	0.35 (0.13)
SEW-WS-MS	9.5	7.5	93.3	1.06	1.80	2.32 (0.33)
	19.0	15.0	92.7	1.38	1.76	3.90 (0.26)
	33.2	26.3	0.0	0.26	1.85	0.27 (n/a)
SEW-WS-A	12.9	8.5	96.5	1.12	4.09	3.37 (0.41)
	25.8	17.0	29.4	0.30	6.11	1.28 (0.26)
	45.1	29.7	16.5	0.35	9.13	0.24 (0.05)
SEW-WS-A-MS	6.6	5.7	88.4	0.92	3.24	2.40 (0.48)
	13.2	11.4	93.9	1.28	3.32	3.82 (0.36)
	23.1	19.8	48.8	1.09	3.01	3.53 (0.36)

[a] Cultures were grown on wood hydrolyzates supplemented with Kp2 medium ingredients. Acetic acid was added in varying amount to give a final acetate concentration of 0.3% (whenever possible). Incubations were carried out at 30°C under finite air conditions with shaking (150 rpm)

[b] Samples were acid hydrolyzates of wood hemicellulose obtained from Table 4

Table 43. Solvent production of *K. pneumoniae* grown on acid hydrolyzed xylan and acid treated xylose[a]

Samples	Initial reducing sugars concn (g/L)	Reducing sugars utilized (%)	Solvents produced (g/L)			
			Ethanol	Acetic Acid	Butanediol	Acetoin
Xylan (5.07 g/100 cc 1% A, 4h)	19.0	91.7	1.27	3.87	4.46 (0.256)	0.19
	37.9	28.5	0.18	4.45	0.63	0.06
Xylan (2.5 g/50 cc 3% A, 2h)	19.8	92.2	1.20	4.81	4.54 (0.249)	ND[b]
	39.5	54.7	1.19	3.02	3.37	0.18
Xylose (2.5 g/50 cc 3% A, 2h)	17.8	92.6	1.70	2.99	5.04 (0.306)	ND
	34.5	11.2	0.20	4.55	1.10	0.03
Xylan (5.0 g/100 cc 3% A, 4h)	25.8	51.2	0.16	4.17	1.65 (0.124)	0.02
	51.6	35.7	0.24	4.96	0.55	0.02
Xylose (5.05 g/100 cc 3% A, 4h)	22.0	93.9	1.74	2.81	5.07 (0.245)	ND
	44.1	23.1	0.25	5.77	0.41	0.02
Xylan (2.5 g/100 cc 3% A, 2h)	17.5	91.2	1.93	3.27	6.20 (0.388)	ND
	35.0	39.1	0.02	4.06	0.13	ND
Xylan (5.0 g/560 cc 3% A, 4h)	18.4	69.0	0.88	4.27	2.61 (0.206)	ND
	36.8	2.7	0.02	4.06	0.13	ND
Xylose (5.0 g/560 cc 3% A, 4h)	22.6	56.9	0.16	4.83	0.97 (0.075)	0.07
	45.1	52.1	0.15	4.31	0.25	ND

[a] Cultures were grown on acid treated samples (obtained from Table 5) supplemented with Kp2 medium ingredients. Incubations were carried out under finite air conditions at 30°C with shaking (150 rpm)

Table 44. Utilization of acid hydrolyzed wood hemicellulose by *K. pneumoniae* for the production of butanediol[a]

Samples	Substrate conc'n at hydrolysis (%)	Initial reducing sugars conc'n (g/L)	Reducing sugars utilized (%)	Solvents present (g/L)				
				Ethanol	Acetic Acid	Butanediol		Acetoin
Xylan	5	16.3	93.6	4.24	0.64	2.91	(0.19)[b]	ND[c]
		32.6	66.9	7.35	0.31	3.84	(0.18)	0.01
	10	32.0	67.9	2.83	0.24	4.21	(0.19)	ND
		64.1	3.8	2.32	1.23	0.75		ND
SEW-WS 15S	5	11.4	91.8	0.81	2.67	1.94	(0.19)	0.29
		22.8	29.7	0.20	3.88	0.03		0.03
	10	23.8	28.5	2.66	4.74	0.65		0.06
		47.7	15.5	4.58	9.11	0.22		0.03
SEW-WS 30S	5	11.7	89.2	0.97	2.71	1.77	(0.17)	0.02
		23.5	22.9	0.18	4.76	0.03		0.05
	10	23.3	23.5	1.00	5.27	0.20		ND
		46.6	5.2	2.00	11.52	0.12		0.04
SEW-WS 60S	5	11.7	80.7	3.37	2.86	1.44	(0.15)	0.02
		23.3	8.8	5.84	4.31	0.23		ND
	10	23.4	28.5	1.32	4.52	0.21		ND
		46.9	9.1	2.30	8.42	0.13		ND

[a] Cultures were grown on wood hemicellulose hydrolyzates (obtained after treatment as stated in Table 6) supplemented with Kp2 medium ingredients and incubated under finite air conditions at 30°C with shaking (150 rpm)

[b] Butanediol yield (g diol/g sugar utilized)

[c] Not detected

Table 45. Butanediol production of *K. pneumoniae* grown on acid hydrolyzed wood hemicellulose[a]

Samples	Initial Monosaccharide conc'n (g/L)	Monosaccharides utilized (%)	Solvents produced (g/L)			
			Ethanol	Acetic acid[b]	Butanediol	Acetoin
SEW-WS 15S	22.5	32.0	0.38	3.30	1.27 (0.18)[c]	0.40
SEW-WS 30S	21.3	100.0	2.93	1.70	9.90 (0.46)	0.18
	39.9	27.8	0.24	3.51	1.38 (0.12)	0.08
SEW-WS 60S	18.5	100.0	2.68	2.07	9.00 (0.49)	0.05
	34.7	23.3	0.86	3.64	3.91 (0.48)	0.13

[a] Cultures were grown on wood hemicellulose hydrolyzates obtained after treatment as described in Table 7. Medium was supplemented with Kp2 ingredients and incubated under finite air conditions at 30°C (150 rpm shaking).

[b] Initial acetic acid concentration was made to 5.0 g/L

[c] Butanediol yield (g/g sugar utilized)

Table 46. Solvent production of *K. pneumoniae* grown on acid hydrolyzed wood cellulose and hemicellulose[a]

Samples	Initial sugar conc'n (g/L)	Sugar utilization (%)	Solvents produced (g/L) Ethanol	Acetic Acid	Butanediol	
SEW-15S-WI 72%A, IR45	10	97.8	1.36	2.61	4.66	(0.476)[c]
	20	90.8	2.61	1.20	9.83	(0.541)
	40	68.2	4.75	0.11	16.84	(0.617)
SEW-30S-WI 72%A, IR45	10	98.0	1.08	2.49	3.55	(0.362)
	20	89.2	2.21	1.69	7.83	(0.439)
	40	81.1	5.86	0.12	20.01	(0.617)
SEW-60-WI 72%A, IR45	10	99.1	0.88	1.07	1.21	(0.122)
	20	98.9	1.15	0.90	3.73	(0.189)
	40	96.2	2.17	0.25	8.84	(0.230)
Holocellulose 72%A, IR45	10	95.7	1.65	2.33	6.06	(0.633)
	20	97.1	3.73	0.64	11.31	(0.583)
	40	80.5	6.07	ND[b]	18.55	(0.576)
Aspen 3%A, IR45	10	94.2	1.35	2.99	3.38	(0.359)
	20	91.5	2.81	2.70	6.50	(0.355)
Holocellulose 3%A, NH_4OH	10	87.0	1.37	5.18	2.44	(0.280)
	20	7.9	0.48	7.78	0.15	(0.009)
Holocellulose 3%A, IR45	10	94.7	1.28	3.06	2.62	(0.277)

[a] Cultures were grown on wood substrates (prepared as described in Table 8) supplemented with Kp2 medium ingredients. Fermentation was carried out under finite air conditions with shaking (150 rpm)

[b] Not detected

[c] Butanediol yield (g diol per g sugar consumed)

Table 47. Butanediol production of K. pneumoniae grown on enzymatically hydrolyzed wood substrates[a]

Substrate	Initial sugar conc'n (g/L)	Sugar utilization (%)	Solvents produced (g/L)		
			Ethanol	Acetic Acid	Butanediol
Holocellulose-5%	9.1	84.1	0.15	2.86	1.75
-10%	9.9	94.4	0.20	4.12	1.72
SEW-WI-15S-5%	4.6	88.9	0.15	2.43	0.36
-10%	8.7	90.5	0.14	2.86	1.63
SEW-WI-30S-5%	1.5	87.0	0.09	1.47	0.09
-10%	6.4	92.1	0.15	2.06	2.93
SEW-WI-60S-5%	1.3	91.5	0.15	2.43	0.36
-10%	3.4	82.5	0.11	1.56	0.02
Xylan 5%	9.8	80.6	0.48	3.24	2.07
10%	8.8	78.0	0.53	2.84	1.97
	17.6	70.0	1.37	2.45	5.05
SEW-WS-15S	9.1	83.6	0.62	3.93	1.48
	18.2	59.1	0.93	5.18	2.62
SEW-WS-30S	10.3	71.3	0.91	4.46	1.30
	20.6	0.0	0.95	5.95	0.05
SEW-WS-60S	9.7	62.1	0.42	3.56	0.52
	19.4	6.1	0.27	4.20	0.03
	38.8	17.8	0.41	9.09	0.0

[a] Cultures were grown on wood substrates hydrolyzed by enzyme (Table 17) supplemented with Kp2 medium. Incubation was carried out under finite air conditions at 30°C (150 rpm shaking)

Table 48. Fermentability of enzymatically hydrolyzed xylan by *K. pneumoniae*[a]

Hydrolysis conditions	Duration of Hydrolysis(d)	Inoculum size (%)	Reducing sugars utilized		Solvents produced (g/L)		
			(g/L)	(%)	Ethanol	Acetic Acid	Butanediol
A-5%	1	2	8.5	69.7	1.17	3.75	1.60
		5	7.3	59.8	1.32	4.01	1.88
	2	2	9.5	72.5	1.50	4.17	3.63
		5	9.3	71.0	1.54	3.90	3.21
A-10%	1	2	9.0	70.9	1.37	3.55	2.55
		5	9.4	74.0	1.45	3.47	2.91
	2	2	10.9	75.7	1.56	3.18	3.98
		5	10.8	75.0	1.41	2.98	3.95
B-5%	1	2	3.4	19.9	0.36	4.08	0.83
		5	3.1	18.1	0.38	4.24	0.41
	2	2	17.1	85.1	1.91	2.85	5.30
		5	16.0	79.6	1.78	3.25	5.53
B-10%	1	2	5.2	28.1	ND	4.39	0.64
		5	6.9	37.3	0.38	3.69	1.16
	2	2	16.8	85.3	1.47	3.74	6.43
		5	17.5	88.8	1.61	3.20	6.53
C-5%	1	2	13.6	78.2	1.74	3.83	3.50
		5	12.6	72.4	1.76	3.29	3.93
	2	2	14.9	77.6	2.10	2.67	6.00
		5	16.7	87.0	1.99	3.55	5.88
C-10%	1	2	12.9	72.1	0.39	3.72	3.93
		5	13.1	73.2	1.72	3.34	3.47
	2	2	9.0	44.5	0.86	4.11	3.20
		5	13.1	64.9	1.56	3.44	4.33

[a] Cultures were grown on xylan preparation after various enzymatic hydrolysis (Table 19), supplemented with Kp2 medium and incubated under finite air conditions at 30^{o}C with shaking (150 rpm).

Table 49. Fermentability of 5% xylan preparations after enzymatic hydrolysis[a]

Hydrolysis conditions	Inoculum size (%)	Reducing sugars utilized		Solvents produced (g/L)			
		(g/L)	(%)	Ethanol	Acetic Acid	Butanediol	Acetoin
5%A-1d	2	9.8	59.8	2.07	0.66	2.56	0.09
	5	9.9	60.4	2.12	0.68	3.03	0.09
-3d	2	14.7	68.7	1.92	2.14	4.37	0.04
	5	14.9	69.6	1.86	1.08	5.11	0.04
10%A-1d	2	15.6	78.0	1.70	3.09	3.22	0.25
	5	15.9	79.5	1.75	3.06	3.31	0.24
-3d	2	12.4	59.0	1.45	2.34	4.96	0.27
	5	10.7	51.0	1.15	2.70	4.21	0.20
5%B-1d	2	10.6	73.1	1.95	3.07	4.70	0.24
	5	10.8	74.5	2.00	3.00	4.84	0.25
-3d	2	10.1	47.4	1.38	2.49	5.67	0.39
	5	14.9	70.0	1.64	2.50	6.78	0.39
10%B-1d	2	20.2	81.5	0.82	3.57	4.22	0.16
	5	21.6	87.1	0.87	4.30	5.52	0.57
-3d	2	18.6	75.0	0.91	3.89	5.76	0.18
	5	19.7	79.4	1.27	4.14	6.27	0.30
5%C-1d	2	16.0	74.1	2.07	3.72	6.02	0.26
	5	16.5	77.1	2.11	3.95	6.41	0.71
-3d	2	9.0	41.3	1.93	2.57	5.68	0.29
	5	16.9	77.5	1.96	2.40	6.23	0.23
10%C-1d	2	23.5	83.6	1.89	2.44	7.79	0.55
	5	24.6	87.5	2.43	2.44	6.14	0.24
-3d	2	19.2	67.4	2.19	1.92	7.77	0.40
	5	19.2	67.4	2.20	1.84	7.83	0.39

[a] Cultures were grown on 5% xylan after enzymatic hydrolysis (Table 20), supplemented with Kp2 medium ingredients and incubated at 30°C under finite air conditions with 150 rpm shaking.

Table 50. Fermentability of 10% xylan preparation after enzymatic hydrolysis[a]

Hydrolysis conditions	Inoculum size (%)	Reducing sugars utilized		Solvents produced (g/L)			
		(g/L)	(%)	Ethanol	Acetic Acid	Butanediol	Acetoin
5%A-1d	2	18.9	70.0	1.86	1.80	5.24	0.05
	5	19.0	70.4	2.28	1.74	5.94	0.08
-3d	2	16.6	58.7	2.14	2.84	6.21	0.13
	5	15.5	54.8	2.42	3.00	7.15	0.11
10%A-1d	2	26.7	73.2	2.27	1.72	6.10	0.07
	5	28.1	77.0	2.67	2.14	7.71	0.08
-3d	2	6.5	16.6	0.02	3.64	0.70	ND
	5	19.6	50.0	2.31	2.66	7.26	0.08
5%B-1d	2	21.6	89.6	ND	2.06	0.94	0.01
	5	21.6	89.6	ND	2.06	0.39	ND
-3d	2	0.0	0.0	0.01	3.41	0.06	ND
	5	0.0	0.0	0.01	3.26	0.08	ND
10%B-1d	2	20.6	42.9	0.28	3.18	2.85	0.07
	5	19.0	39.6	0.26	3.23	2.51	0.05
-3d	2	18.7	38.8	0.05	2.54	0.23	0.06
	5	12.5	25.9	0.01	3.66	ND	0.03
5%C-1d	2	13.6	54.8	2.89	1.66	9.50	0.07
	5	15.7	63.3	3.32	1.39	11.00	0.10
-3d	2	10.5	28.5	0.21	3.61	0.32	ND
	5	10.0	27.1	0.04	3.40	0.23	ND
10%C-1d	2	16.8	34.0	0.04	3.59	1.38	0.06
	5	17.6	35.6	0.05	3.58	0.96	0.02
-3d	2	6.9	13.9	0.04	3.05	0.28	0.22
	5	14.2	28.6	0.01	3.38	0.51	ND

[a] Cultures were grown on enzymatically hydrolyzed xylan (Table 20) supplemented with Kp2 medium ingredients. Fermentation was carried out at 30°C under finite air conditions with shaking (150 rpm).

Table 51. Utilization of enzymatically hydrolyzed wood hemicellulose for butanediol production by *K. pneumoniae*[a]

Substrate	Initial reducing sugars conc'n (g/L)	Reducing sugars utilized (%)	Solvents produced (g/L)			
			Ethanol	Acetic Acid	Butanediol	Acetoin
Xylan	9.27	84.1	0.78	2.02	1.48	0.10
	22.68	86.2	2.13	1.71	3.84	0.12
	50.48	76.5	3.00	0.11	6.87	0.07
SEW-WS-15S	8.92	80.5	0.89	2.89	1.49	0.03
	31.50	51.4	0.95	2.39	1.11	0.05
	39.90	0.0	0.22	6.94	0.29	0.04
SEW-WS-30S	9.43	75.9	0.76	2.79	1.05	0.17
	20.45	14.7	0.21	4.87	0.27	0.43
	40.31	7.7	ND	8.22	0.07	0.01
SEW-WS-60S	9.52	73.4	0.82	3.47	0.81	0.02
	18.96	18.5	0.25	3.80	0.20	0.04
	46.81	23.5	0.24	5.30	0.14	0.02

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[a] Cultures were grown on wood hemicellulose hydrolyzed by *T. harzianum* E58 xylanase (Table 21) at 50°C, pH 5.0 for 2 days. The medium was supplemented with Kp2 ingredients and incubated at 30°C under finite air conditions.

Table 52. Fermentation of wood hemicellulose after enzymatic hydrolysis by *K. pneumoniae*[a]

Substrate	Initial Reducing Sugars Concentration (g/L)	Reducing Sugars Utilized (%)	Solvents Produced (g/L)			
			Ethanol	Acetic Acid	Butanediol	Acetoin
Xylan	34.3	62.8	0.35	2.89	0.96	0.05
	66.8	59.1	0.03	3.63	0.19	ND[b]
SEW-WS-15S	33.1	47.7	0.02	4.54	0.14	0.04
	72.8	41.2	0.02	7.71	0.09	0.02
SEW-WS-30S	38.8	47.6	0.02	5.47	0.13	0.02
	74.6	46.1	0.02	8.27	0.03	0.02
SEW-WS-60S	36.4	50.8	0.02	5.83	0.12	0.03
	86.2	52.3	0.02	7.53	0.12	0.03

[a] Cultures were grown on wood hemicellulose hydrolyzates (Table 23) supplemented with Kp2 medium. Incubation was carried out under finite air conditions at 30°C with shaking (150 rpm)

[b] Not detected

Table 53. Solvent production by K. pneumoniae grown on wood hemicellulose hydrolyzates[a]

Substrate (and conc'n)	Inoculum size (%)	Reducing sugars utilized (g/L)	Reducing sugars utilized (%)	Solvents produced (g/L) Ethanol	Acetic Acid	Butanediol	
Xylan 2%	2	6.93	77.0	1.66	7.34	3.00	(0.433)[b]
	5	6.01	66.8	0.59	7.65	3.57	(0.594)
Xylan 4%	2	12.74	81.9	3.08	4.68	7.36	(0.578)
	5	9.91	63.7	0.67	7.84	6.36	(0.642)
SEW-WS-15S-2%	2	7.89	80.2	0.62	2.96	0.87	(0.110)
	5	8.47	86.1	1.42	4.18	4.50	(0.531)
SEW-WS-30S-2%	2	0.00	0.0	0.17	1.45	0.11	(n/a)
	5	8.67	85.1	1.03	4.29	4.34	(0.501)
SEW-WS-30S-4%	2	16.90	88.6	0.68	2.60	0.83	(0.049)
	5	7.28	38.1	1.25	6.20	3.40	(0.467)
SEW-WS-60S-2%	2	0.00	0.0	0.20	3.34	0.08	(n/a)
	5	8.36	81.9	0.66	4.20	3.66	(0.438)
SEW-WS-60S-4%	2	0.23	1.2	0.25	5.60	0.06	(0.261)
	5	6.74	36.0	0.53	5.09	2.18	(0.323)

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[a] Cultures were grown on wood hemicellulose hydrolyzed by T. E58 culture filtrates (Table 24) supplemented by Kp2 medium ingredients and incubated at 30°C with shaking (150 rpm)

[b] Butanediol yield (g/g sugar consumed)

Table 54. Solvent production by *K. pneumoniae* grown on enzymatically hydrolyzed wood cellulose[a]

Substrate	Initial Reducing sugars conc'n (g/L)	Reducing sugars utilized (%)	Solvents produced (g/L)				
			Ethanol	Acetic Acid	Butanediol		Acetoin
Solka floc	24.5	94.9	2.09	3.49	6.47	(0.278)[b]	0.53
	40.0	82.6	3.19	2.49	10.72	(0.324)	0.44
SEW-WI-15S	9.6	91.2	0.65	4.07	5.25	(0.600)	0.04
	15.1	83.7	1.02	4.81	4.65	(0.368)	0.23
SEW-WI-30S	8.8	93.1	0.47	4.36	3.28	(0.400)	0.08
	11.7	92.6	0.60	4.22	3.59	(0.331)	0.12
SEW-WI-60S	10.5	91.8	0.50	4.21	3.11	(0.323)	0.15
	10.5	91.4	0.69	4.07	3.45	(0.359)	0.08

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[a] Cultures were grown on wood cellulose hydrolyzates (Table 25) supplemented with Kp2 medium ingredients. Incubations were carried out at 30°C under finite air conditions with shaking (150 rpm)

[b] Butanediol yields (g/g sugar consumed)

Table 55. Solvent production by *C. thermosaccharolyticum* grown in media with modified nitrogen source and reducing agent[a]

Media[b]	Strain ATCC	O.D. (660 nm)	final pH	Glucose used (g/L)	Solvents (g/L) Ethanol	Acetic Acid	Butanol	Butyric Acid
Urea + cysteine	7956	1.2	4.4	10.6	2.38	0.84	0.53	0.52
Urea + thio		1.2	4.3	11.0	2.65	0.69	0.49	0.54
NH_4 + cysteine		1.8	4.4	11.9	1.80	1.18	0.34	1.04
NH_4 + thio		0.6	5.9	14.4	3.31	0.37	0.42	0.21
Urea + cysteine	25773	1.2	4.1	7.3	0.97	1.10	0.25	0.70
Urea + thio		1.2	4.1	6.7	0.69	1.16	0.23	0.72
NH_4 + cysteine		1.6	4.1	13.0	0.59	1.47	0.21	1.22
NH_4 + thio		1.5	4.1	12.5	0.77	1.39	0.21	1.00

[a] Cultures were grown under conditions described in Materials and Methods

[b] Urea and $(NH_4)_2SO_4$ were used as nitrogen source; cysteine-HCl and Na^+ thioglycollate were used as reducing agent

Table 56. Production of solvents by *C. thermosaccharolyticum* (Strain 7956) on sugars present in wood hemicellulose[a]

Sugar	Conc'n (%)	O.D. (660 nm)	Final pH	Solvents (g/L)			
				Ethanol	Acetic Acid	Butanol	Butyric Acid
Glucose	1.5	1.0	4.6	0.66	1.15	0.18	1.33
	3.0	0.3	4.7	0.56	1.06	0.16	0.98
	6.0	0.8	4.3	1.21	1.15	0.23	0.90
Xylose	1.5	0.8	4.6	0.42	0.93	0.15	1.37
	3.0	1.2	4.4	0.61	1.34	0.17	1.25
	6.0	0.2	5.3	0.54	1.40	0.16	1.36
Mannose	1.5	0.1	6.5	0.05	0.24	ND	0.16
	3.0	1.0	4.4	1.13	1.11	0.31	0.95
	6.0	0.9	4.4	0.66	1.07	0.27	1.22
Arabinose	1.5	0.8	4.6	0.40	1.25	0.18	1.70
	3.0	1.0	5.6	0.40	1.42	0.16	1.55
	6.0	0.2	5.6	0.41	1.30	0.16	1.49
Galactose	1.5	0.1	6.4	0.09	0.41	0.09	0.16
	3.0	1.0	4.4	0.99	1.42	0.32	1.51
	6.0	0.7	4.4	0.49	1.09	0.22	1.36
Cello-biose	1.5	1.3	4.6	1.04	1.17	0.29	0.89
	3.0	1.2	4.6	1.12	1.21	0.27	1.05
	6.0	1.1	4.6	1.00	1.31	0.24	1.23

[a] Cultures were grown under conditions described in Materials and Methods

Table 57. Solvent production by *C. thermosaccharolyticum* (strain 7956) grown on sugars prepared separately from medium ingredients[a]

Sugar	Incubation Time (days)	Solvents (g/L)			
		Ethanol	Acetic Acid	Butanol	Butyric Acid
Glucose	2	0.59	1.09	0.13	0.38
	7	0.68	0.74	0.12	0.56
Xylose	2	0.12	0.57	0.03	0.18
	7	0.28	0.51	0.06	0.28
Cellobiose	2	0.31	0.87	0.07	0.27
	7	0.43	0.96	0.08	0.44
Mannose	2	0.41	0.82	0.09	0.43
	7	0.68	0.87	0.12	0.56
Arabinose	2	0.23	0.92	0.05	0.15
	7	0.32	0.72	0.05	0.27
Galactose	2	0.32	0.75	0.05	0.16
	7	0.45	0.83	0.09	0.20

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[a] Cultures were grown in media with 5% sugar levels. Sugars were prepared and autoclaved separately and then mixed with medium ingredients.

Table 58. Solvent production by *C. thermosaccharolyticum* grown on wood hemicellulose hydrolyzates[a]

Wood Samples [a]	Incubation Time (days)	Xylose present (g/L)	Glucose present (g/L)	Solvents (g/L) Ethanol	Solvents (g/L) Acetic Acid	Solvents (g/L) Butyric Acid
1	0	15.6	1.2	0.5	0.49	ND
	2	11.2	4.0	0.20	1.35	0.55
	7	9.9	2.5	0.18	1.69	0.78
2	0	12.1	0.8	1.28	1.38	ND
	2	10.4	2.6	1.33	1.41	0.53
	7	9.4	0.5	1.39	2.52	0.98
3	0	11.7	ND	0.08	2.99	ND
	2	13.7	ND	ND	2.31	0.62
	7	7.5	ND	0.16	4.07	1.02
4	0	7.2	ND	ND	3.57	ND
	2	11.7	ND	0.23	4.03	0.36
	7	3.9	ND	0.35	2.90	0.84
5	0	8.4	ND	0.32	2.46	ND
	2	8.3	ND	0.23	2.57	0.17
	7	5.0	ND	0.11	4.02	0.83
6	0	15.5	ND	ND	4.13	ND
	2	10.4	ND	0.17	3.69	0.13
	7	7.5	ND	0.13	5.70	0.98
7	0	17.5	ND	ND	0.60	ND
	2	17.7	ND	0.17	5.66	0.59
	7	15.3	3.8	0.13	2.17	1.23

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[a] Wood samples used were: (1) SEW-WS-MS; (2) SEW-WS; (3) SEW-WS-A-MS; (4) Aspen (alcohol-benzene extracts, 1h); (5) Aspen (alcohol-benzene extracted, 2h); (6) SEW-WS-A and (7) Xylan (Sigma). All samples were acid hydrolyzed as described in Table 4.

Table 59. Solvent production of *C. acetobutylicum* on sugars present in wood hemicellulose hydrolyzates[a]

Sugar	Conc (%)	O.D. (660 nm)	Solvents (g/L)					
			Ethanol	Acetone	Acetic Acid	Butanol	Butryic Acid	Butanol (autoclaved together)
Glucose	5	3.4	0.26	1.13	1.22	3.02	1.15	4.34
	10	3.3	0.30	1.25	1.38	3.37	0.97	3.32
Xylose	5	1.7	0.18	0.18	1.34	0.51	2.77	0.89
	10	1.9	0.18	0.28	1.65	0.77	2.93	1.43
Cellobiose	5	3.8	0.64	2.41	1.41	5.52	0.84	6.00
	10	3.8	0.59	2.38	1.62	5.33	0.71	5.76
Mannose	5	2.5	0.21	0.58	1.76	1.35	2.04	1.36
	10	3.1	0.31	1.06	1.39	2.92	1.22	1.18
Arabinose	5	5.4	0.75	2.51	1.39	4.33	0.56	3.02
Galactose	5	2.4	0.21	0.37	1.09	0.29	2.59	0.31

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[a] Cultures were grown in CAB medium with various substrates and incubated statically at 37°C for 2 days

Table 60. Effect of various concentrations of MES on sugar utilization and solvent production of *C. acetobutylicum*[a]

MES conc (g/L)	Sugar (3%)	O.D. (660 nm)	Final pH	Sugar used (g/L)	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
0	Glucose	3.6	4.1	20.3	0.36	1.00	1.39	3.74	0.82
0.5		3.4	4.0	9.4	0.15	0.28	1.20	0.93	1.92
1.0		3.4	4.0	7.8	0.20	0.37	1.39	1.53	1.84
1.5		3.4	4.1	6.6	0.20	0.32	1.48	1.05	2.06
0	Xylose	2.6	4.1	8.2	0.13	0.16	1.33	0.41	2.83
0.5		2.8	4.1	5.6	0.15	0.21	1.24	0.48	2.86
1.0		2.1	4.1	3.6	0.14	0.13	1.17	0.34	2.55
1.5		2.5	4.1	6.0	0.13	0.13	1.16	0.36	1.30

[a] Cultures were incubated at 37°C for 72.5 h

Table 61. Acclimatization attempts on *C. acetobutylicum* by serial subculture[a]

Strain (ATCC)	Sugar	# of transfer	O.D. (660 nm)	Final pH	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
2490	Glucose	0	3.8	4.0	0.71	2.23	1.12	5.75	0.46
		1	2.8	3.4	0.18	0.29	1.02	0.81	2.80
		2	-	no growth					
	Xylose	0	2.1	3.8	0.25	0.77	1.57	2.04	1.57
		1	0.4	4.1	0.12	0.21	0.25	0.47	0.18
		2	-	no growth					
824	Glucose	0	3.8	4.0	0.91	2.42	0.72	6.19	0.23
		1	3.4	3.6	0.20	0.38	0.25	1.38	2.24
		2	-	no growth					
	Xylose	0	3.9	3.9	0.60	1.81	0.92	5.46	0.52
		1	2.2	3.6	0.28	0.81	1.12	2.38	1.12
		2	-	no growth					

[a] Cultures were incubated statically at 37°C for 7 days in media containing 5% glucose or 10% xylose. Subculturing was carried out on a weekly basis, using 10% (v/v) inoculum.

Table 62. Solvent production of *C. acetobutylicum* grown on wood hemicellulose hydrolyzates[a]

Wood Samples	Incubation Time (days)	Xylose (g/L)	Glucose (g/L)	Solvents (g/L)				
				Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
1	0	29.3	3.5	<.05	ND	0.92	ND	ND
	7	30.7	5.8	0.08	0.10	0.67	ND	0.28
2	0	22.7	1.5	2.39	ND	2.60	ND	ND
	7	28.9	ND	2.90	0.14	3.57	ND	1.62
3	0	22.0	ND	0.15	ND	5.60	ND	ND
	7	26.3	ND	0.16	0.08	5.86	ND	ND
4	0	7.7	ND	ND	ND	3.57	ND	ND
	7	8.6	ND	0.39	0.05	2.70	ND	0.36
5	0	8.4	ND	0.32	ND	2.46	ND	ND
	7	6.1	2.0	0.07	0.10	4.33	ND	1.31
6	0	24.3	ND	ND	ND	6.50	ND	ND
	7	22.0	ND	0.15	0.13	7.60	ND	1.96
7	0	37.6	ND	ND	ND	0.94	ND	0.16
	7	32.4	ND	0.07	0.06	1.55	ND	0.42

[a] Wood samples used were described previously in Tables 4 and 58. Cultures were incubated at 37°C without shaking.

Table 63. Effect of medium Fe^+ concentration on solvent production by *C. acetobutylicum*[a]

Medium	Substrate	Substrate conc'n (%)	Incubation Time (d)	Growth (O.D. 660 nm)	Sugar utilized (%)	Solvents (g/L)				
						Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
CAM	Glucose	2	2	4.52	54.0	0.16	0.73	1.49	2.14	1.78
			4	3.92	96.0	0.33	1.53	1.23	3.62	0.83
		4	2	7.77	69.0	0.71	2.37	0.59	5.04	0.23
			4	3.03	98.0	1.58	2.89	0.37	6.35	0.18
CAM + Fe	Glucose	4	2	3.20	30.0	0.11	0.53	1.35	1.48	1.42
			4	4.35	93.0	1.15	3.09	0.86	6.58	0.58
Volesky[b]	Glucose	4	2	4.34	19.0	0.17	0.81	1.40	2.19	1.33
			4	7.95	93.0	0.46	1.37	1.50	3.27	1.72
CAM	Xylose	4	2	1.91	0.0	0.12	0.14	1.08	0.31	1.71
			4	1.71	0.0	0.08	0.09	1.30	0.37	2.44
CAM + Fe	Xylose	4	2	1.69	0.0	0.07	0.05	1.16	0.14	2.01
			4	0.97	45.0	0.08	0.16	1.39	0.32	2.65
Volesky	Xylose	4	2	3.66	11.0	0.15	0.42	1.51	1.13	2.06
			4	2.85	32.0	0.11	0.04	1.61	0.33	3.12

[a] Cultures were incubated statically at 37°C in CAB medium with 0.5% cysteine-HCl under N_2 headspace

[b] Medium with high ferrous concentration, as described by Volesky *et al* (1980)

Table 64. The influence of inoculum age on solvent production of C. *acetobutylicum*[a]

Substrate	Inoculum Age	Incubation Time (d)	O.D. (660 nm)	% C utilized	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	RCM	2	7.8	62.5	0.02	1.08	0.62	3.87	0.54
		7	6.8	67.8	0.04	1.25	0.69	4.08	0.39
	CAM 1d	2	6.5	47.5	0.27	0.90	0.83	3.32	0.65
		7	5.8	42.8	0.33	1.20	1.00	3.67	0.30
	CAM 2d	2	6.6	38.9	0.23	0.66	1.20	2.76	0.70
		7	5.3	38.0	0.57	1.58	0.86	4.02	0.37
	CAM 3d	2	6.6	36.5	0.20	0.77	1.26	2.88	0.46
		7	5.7	42.4	0.58	1.29	0.65	4.34	0.35
Xylose	RCM	2	3.7	23.5	0.13	0.21	1.28	0.61	2.19
		7	2.5	15.0	0.06	0.44	1.64	0.72	2.36
	CAM 1d	2	3.8	25.6	0.11	0.32	1.29	1.44	1.36
		7	3.2	22.3	0.21	0.46	1.83	1.89	1.26
	CAM 2d	2	4.0	10.6	0.40	0.12	1.15	3.06	0.52
		7	3.2	11.8	0.29	0.65	1.77	1.97	1.72
	CAM 3d	2	4.0	13.8	0.12	0.55	1.93	1.28	1.78
		7	3.1	9.1	0.28	0.58	1.68	1.77	1.95

[a] Cultures were grown in CAM with 0.5% cysteine-HCl. Incubations were carried out statically at 37°C under N_2 headspace.

Table 65. Solvent production of *C. acetobutylicum* grown anaerobically under various headspace gasing and pressurizing[a]

Substrate (4%)	Flushing (2X/day)	Pressurizing	O.D. (660 nm)	C. utilization (%)	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	(-)	(-)	4.31	11.6	0.26	0.30	3.18	1.44	3.02
	(+)	(-)	4.54	10.6	0.28	0.35	2.23	1.48	3.06
	(+)	(+)	4.58	11.5	0.27	0.36	2.21	1.41	2.94
	(+)	(++)	4.35	16.0	0.30	0.43	2.30	1.76	2.80
Xylose	(-)	(-)	4.74		0.26	0.28	1.86	1.17	3.46
	(+)	(-)	4.43	18.0	0.26	0.27	2.09	1.20	3.45
	(+)	(+)	4.65	13.2	0.28	0.33	1.90	1.10	3.19
	(+)	(++)	4.41	10.0	0.25	0.30	1.97	1.26	3.21

[a] Cultures were grown in CAM medium with 0.5% cysteine-HCl. Incubations were carried out statically at 37°C under N_2 atmosphere. Flushing, when carried out, was performed twice daily with nitrogen gas in the headspace for 30 sec. Pressurizing was achieved at two arbitrary levels: with vial pressure at equilibrium with external gas flow rate of 1 L/min and 3 L/min, as indicated by (+) and (++), respectively.

Table 66. The influence of inoculum preparation on acetone-butanol fermentation by _C_. _acetobutylicum_[a]

Substrate	Stock age	Inoculum Age	O.D. (660 nm)	% C utilized	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	1 week	1d	3.06	14.8	0.23	0.84	1.87	2.56	1.19
		2d	3.36	26.7	0.25	0.82	1.47	2.78	1.40
		3d	4.49	33.4	0.32	1.19	1.13	3.58	0.72
		4d	6.83	65.1	0.65	1.38	0.55	4.95	0.30
	2 weeks	1d	6.26	50.5	0.60	2.03	0.79	4.92	0.32
		2d	6.59	58.8	0.63	1.85	0.61	5.00	0.34
		3d	6.35	57.2	0.68	1.89	0.69	4.95	0.36
		4d	6.28	64.8	0.72	1.99	0.59	5.15	0.37
	3 weeks	2d	4.47	19.8	0.15	0.11	1.25	0.85	2.91
Xylose	1 week	1d	2.96	9.6	0.20	0.35	1.79	1.31	2.24
		2d	3.95	40.0	0.22	0.57	1.47	2.23	1.72
		3d	4.31	24.8	0.18	0.28	1.98	1.21	2.64
		4d	5.25	40.9	0.46	1.44	1.33	4.22	0.53
	2 weeks	1d	3.97	26.6	0.25	0.68	1.88	2.62	1.64
		2d	5.14	52.9	0.57	1.70	1.09	4.75	0.70
		3d	3.74	44.7	0.40	1.25	1.45	3.76	0.85
		4d	3.91	32.6	0.40	1.24	1.67	3.91	0.88
	3 weeks	2d	4.24	13.4	0.20	0.19	1.60	0.82	3.39

[a] Inocula were grown in CAM with 0.5% cysteine-HCl. Test cultures were grown in CAM without added cysteine and incubated for 2 days under non-shaking conditions at 37°C under H_2 atmosphere.

Table 67. Acetone-butanol fermentation by *C. acetobutylicum* using inocula grown in the absence of cysteine[a]

Substrate (4%)	Stock age	Inoculum Age (d)	O.D (660 nm)	% C utilized	Solvents (g/L) Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	1 week	1	5.50	42.9	0.60	2.13	1.08	5.20	0.37
		2	6.23	63.5	0.64	1.83	0.63	4.97	0.37
		3	6.15	58.7	0.60	1.92	1.00	5.12	0.53
		4	5.58	78.5	0.85	2.13	0.56	5.48	0.25
	2 weeks	1	6.34	54.0	0.57	2.00	1.02	4.92	0.31
		2	6.20	71.8	0.64	1.87	0.65	5.06	0.35
		3	6.08	77.0	0.78	2.20	0.64	5.54	0.30
		4	5.12	91.4	1.03	1.83	0.26	5.16	0.16
Xylose	1 week	1	3.92	30.0	0.25	0.29	1.86	1.70	2.52
		2	3.73	38.2	0.28	0.42	1.62	1.71	2.91
		3	4.11	34.5	0.27	0.58	1.68	2.27	2.10
		4	4.50	30.2	0.30	0.57	1.67	2.16	2.23
	2 weeks	1	3.70	43.6	0.29	0.58	1.90	2.42	1.99
		2	3.88	37.1	0.30	0.45	1.72	1.53	3.00
		3	4.30	31.5	0.29	0.57	1.70	2.17	2.21
		4	4.51	31.3	0.33	0.58	1.75	2.20	2.27

[a] Both cultures used as inocula or test were grown in CAM without cysteine and incubated statically under hydrogen at 37°C for 2 days.

Table 68. The influence of substrates in inoculum media on the solvent production by *C. acetobutylicum*[a]

Substrate in inoculum	Inoculum age (d)	Incubation Time (d)	O.D. (660 nm)	% C utilized	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	2	2	3.79	34.3	0.39	1.01	1.61	3.34	1.29
		5	2.34	46.4	0.44	1.08	1.75	3.48	1.21
	3	2	4.02	37.9	0.44	1.15	1.49	3.51	0.86
		5	2.54	38.0	0.41	1.11	1.63	3.44	1.17
	4	2	4.28	32.1	0.49	1.23	1.52	3.80	0.69
		5	2.68	43.2	0.55	1.62	1.43	4.40	0.77
Xylose	2	2	1.76	8.5	0.09	0.05	1.25	0.36	2.68
		5	1.37	5.0	0.09	0.12	1.54	0.54	2.86
	3	2	3.29	16.0	0.16	0.10	1.29	0.26	3.39
		5	1.85	16.2	0.17	0.09	1.32	0.30	3.55
	4	2	0.02	0.0	0.03	0.03	0.11	0.07	0.23
		5	0.09	0.0	0.03	0.04	0.20	0.14	0.16

[a] All cultures were grown on 4% xylose in CAM without cysteine and incubated statically at 37°C under H_2 atmosphere

Table 69. The effect of medium storage (by refrigeration) on acetone-butanol fermentation[a]

Substrate	Inoculum Age (d)	Incubation Time (d)	O.D. (660 nm)	% C utilized	Solvents (g/L)				
					Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glucose	2	2	5.69	73.2	0.77	1.83	0.60	5.05	0.45
		5	4.82	68.2	0.79	1.84	0.60	5.01	0.49
	3	2	6.45	75.0	0.79	1.89	0.61	5.20	0.52
		5	4.39	74.3	0.74	1.95	0.66	5.16	0.48
	4	2	6.45	75.1	0.74	1.89	0.58	5.26	0.33
		5	5.02	72.8	0.76	1.89	0.65	5.11	0.34
Xylose	2	2	3.64	6.2	0.33	0.77	1.78	2.87	1.70
		5	2.78	24.5	0.35	0.93	1.83	3.17	1.58
	3	2	4.49	28.8	0.32	0.70	1.66	2.56	2.18
		5	2.89	26.8	0.33	0.85	1.74	2.90	1.82
	4	2	3.95	27.8	0.32	0.65	1.74	2.45	2.24
		5	2.71	28.7	0.27	0.60	1.65	2.34	1.97

[a] Cultures were grown in CAM medium previously stored at $4^{o}C$ for 7 days. Incubations were carried out statically at $37^{o}C$ under H_2 atmosphere.

ble 70. The effect of heat-shocked inoculum preparation on acetone-butanol fermentation by C. acetobutylicum[a]

noculum	Inoculum Age (d)	Medium substrate	Incubation Time (d)	O.D. (660 nm)	% C utilized	Solvents (g/L)				
						Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
at-shocked	2	Glucose	2	6.13	70.1	0.68	1.85	0.48	5.22	0.32
			5	4.90	74.2	0.68	1.77	0.56	5.14	0.35
	3		2	5.70	74.3	0.70	1.82	0.58	4.96	0.48
			5	4.42	78.1	0.68	1.84	0.58	5.24	0.45
	4		2	5.70	74.2	0.84	1.92	0.47	5.63	0.30
			5	3.58	77.8	0.75	1.82	0.51	5.06	0.39
	2	Xylose	2	4.33	39.6	0.46	1.35	1.34	4.35	0.56
			5	2.04	38.1	0.52	1.49	1.49	4.60	0.67
	3		2	4.23	46.7	0.37	0.70	1.74	2.71	2.08
			5	2.85	31.2	0.32	0.69	1.75	2.74	2.23
	4		2	4.05	26.5	0.47	1.07	1.52	3.51	0.94
			5	2.54	26.5	0.37	0.94	1.61	3.04	1.28
ontrol	2	Glucose	2	5.13	78.7	0.77	2.00	0.52	5.56	0.32
			5	3.45	78.9	0.78	1.91	0.56	5.45	0.31
	3		2	5.62	82.6	0.79	1.92	0.48	5.37	0.29
			5	3.95	84.2	0.74	1.99	0.53	5.58	0.27
	4		2	6.12	70.3	0.73	1.78	0.63	5.30	0.33
			5	4.82	78.6	0.75	2.04	0.72	5.42	0.43
	2	Xylose	2	3.79	34.3	0.39	1.01	1.61	3.34	1.29
			5	2.34	46.4	0.44	1.08	1.75	3.48	1.21
	3		2	4.02	37.9	0.44	1.15	1.49	3.51	0.86
			5	2.54	38.0	0.41	1.11	1.63	3.44	1.17
	4		2	4.28	32.1	0.49	1.23	1.52	3.80	0.69
			5	2.68	43.2	0.55	1.62	1.43	4.40	0.77

[a] Heat shock was carried out by boiling inocula cultures (in CAM without cysteine) for 90 sec., cooled rapidly to 37°C and then incubated statically at 37°C for 2-4 days. The test cultures were initiated by using 5% inoculum (v/v) and then incubated under the same conditions.

able 71. The influence of heat shock of inocula on acetone-butanol fermentation by C. acetobutylicum[a]

Inoculum	Inoculum Age (d)	Medium substrate	Incubation Time (d)	O.D. (660 nm)	% C utilized	Solvents (g/L) Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
eat-shocked	2	Glucose	2	0.02	1.2	ND	0.03	0.10	0.11	0.25
			5	0.03	0.0	0.04	0.03	0.18	0.11	0.16
	3		2	0.04	0.0	0.03	0.04	0.16	0.16	0.27
			5	0.03	0.0	0.04	0.03	0.05	0.07	0.11
	4		2	0.03	0.0	0.03	0.03	0.08	0.06	ND
			5	0.02	0.0	0.02	0.04	0.16	0.11	0.09
	2	Xylose	2	0.02	0.0	0.02	0.02	0.03	0.13	0.08
			5	0.04	0.0	ND	0.02	0.18	0.15	0.10
	3		2	0.02	0.0	0.03	0.04	0.03	0.12	0.11
			5	0.04	0.0	0.03	0.03	0.04	0.08	0.07
	4		2	0.03	0.0	ND	0.02	0.08	0.08	ND
			5	0.02	1.5	0.01	0.03	0.13	0.11	ND

Heat shock procedure was the same as described in Table 70 except that inocula cultures were grown in CAM with 0.5% cysteine-HCl. Incubations of test cultures were carried out at 37^{o}C without shaking under hydrogen atmosphere.

Table 72. The influence of incubation temperature on acetone-butanol fermentation[a]

Substrate	T^{o}	OD	C. utilized (%)	Solvents (g/L)				
				Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Glu	37	4.92	71.6	0.86	2.10	0.57	5.73	0.26
	30	6.61	91.2	0.90	2.49	0.64	8.14	0.31
Xyl	37	3.19	33.5	0.21	0.36	1.78	2.69	1.05
	30	3.78	31.8	0.18	0.26	1.71	1.83	3.07

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[a] Cultures were grown in CAM medium without cystein-HCl and incubated statically under hydrogen atmosphere for 2 days.

Table 73. Utilization of sugars and uronic acids present in wood hemicellulose for the production of acetone and butanol[a]

Substrates	Substrate utilized (g/L)	Solvents (g/L)				
		Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
D-glucose	36.1	1.85	3.56	0.86	9.31	0.30
D-mannose	38.3	1.08	3.32	1.33	10.22	0.95
D-galactose	13.9	0.17	0.03	1.45	0.39	3.79
D-cellobiose	35.5	0.85	2.87	1.71	9.04	1.34
D-xylose	15.1	0.36	0.72	1.98	2.36	2.37
L-arabinose	29.0	0.87	3.38	2.04	7.12	2.01
D-glucuronic acid	ND[b]	0.18	0.59	2.60	1.23	2.22
D-galacturonic acod	ND	0.03	0.01	0.15	0.23	0.34

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[a] Cultures were grown in CAM medium without cysteine and incubated statically under H_2 at 37°C for 2-5 days.

[b] Not determined

Table 74. Acetone-butanol production by *C. acetobutylicum* grown on acid hydrolyzed wood hemicellulose[a]

Substrates	Initial Sugar concn (g/L)	% sugar utilization	Solvents produced (g/L)				
			Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
SEW-WS 15S 5%	10.2	81.9	0.32	0.23	3.73	0.59	2.67
	20.3	93.4	0.31	0.21	4.98	0.44	1.75
10%	10.5	52.6	1.51	0.34	4.54	0.56	2.20
	21.1	4.3	2.47	0.37	6.89	0.47	0.66
	31.2	0.0	3.52	0.42	9.90	0.51	0.39
SEW-WS 30S 5%	10.1	81.2	0.32	0.25	4.56	0.75	2.55
	20.2	77.2	0.25	0.16	5.80	0.42	0.79
10%	10.7	52.3	0.69	0.31	6.11	0.62	2.08
	21.3	2.6	0.97	0.25	7.97	0.51	0.60
	29.4	0.0	1.27	0.26	10.64	0.51	0.35
SEW-WS 60S 5%	10.4	83.9	2.03	0.31	4.63	0.58	2.44
	20.8	17.6	4.09	ND[b]	5.34	0.41	0.76
10%	10.7	60.0	0.77	0.22	6.11	0.44	2.25
	21.3	0.0	1.21	0.22	7.59	0.57	0.61
	31.7	0.0	1.64	0.27	10.34	0.53	0.36
Xylan 5%	10.2	60.0	1.59	0.26	1.35	0.35	2.32
	20.3	0.0	2.71	0.41	1.32	0.34	0.34
10%	10.3	69.3	0.41	0.22	3.24	0.51	2.01
	20.6	22.3	0.53	0.20	1.48	0.37	0.78
	39.6	30.8	0.79	0.24	0.84	0.34	0.32

[a] Cultures were grown anaerobically under H_2 with wood hemicellulose hydrolyzed by acid and neutralized by NH_4OH (Table 6). Incubations were carried out statically at 37°C.

[b] Not determined.

Table 75. Solvent production by *C. acetobutylicum* grown on acid-hydrolyzed wood substrates[a]

Samples	Initial sugar concn (g/L)	Sugar utilization (%)	Solvents produced (g/L)				
			Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
SEW-15S-WI	10.4	95.5	0.32	0.31	1.75	1.63	1.94
72%A, IR 45	20.8	97.1	0.50	0.88	2.08	3.66	1.67
	38.7	95.6	0.98	2.70	1.12	7.77	1.04
SEW-30S-WI	10.2	95.2	0.39	0.46	1.69	2.04	1.59
72%A, IR 45	20.4	96.7	0.66	1.37	1.46	4.74	0.94
	40.7	88.5	1.14	3.03	0.97	8.98	0.53
SEW-60S-WI	9.9	95.5	0.34	0.52	1.48	2.16	1.40
72%A, IR 45	19.8	96.8	0.64	1.31	1.17	4.95	0.99
Holocellulose	10.2	95.0	0.32	0.24	1.53	0.98	1.92
72%A, IR 45	20.4	95.8	0.54	1.08	1.96	3.92	1.55
Aspen	10.6	90.8	0.28	0.11	1.57	0.50	2.81
3%A, IR 45	20.3	91.3	0.49	0.65	2.70	2.79	2.77
Holocellulose	10.7	0.0	0.46	0.19	3.22	0.38	0.55
3%A, NH_4OH	17.1	54.7	ND	0.04	2.76	0.26	0.13
Holocellulose	10.9	90.5	0.35	0.23	2.47	0.78	2.41
3%A, IR 45	19.9	91.5	0.48	0.82	3.00	2.97	2.35

[a] Cultures were grown on wood substrates hydrolyzed by acid (Table 8), supplemented with CAB medium ingredients and incubated under hydrogen at 37°C without shaking.

Table 76. Solvent production of *C. acetobutylicum* grown on enzymatically hydrolyzed wood substrates[a]

Substrates	Initial sugar concn (g/L)	Sugar utilization (%)	Solvents (g/L)				
			Ethanol	Acetone	Acetic Acid	Butanol	Butyric Acid
Holocellulose - 5%	8.7	0.0	ND[b]	0.03	0.95	0.37	0.09
- 10%	8.9	57.3	ND	0.02	0.90	0.28	0.27
SEW-WI-15S-10%	10.1	97.0	0.24	0.11	1.38	0.41	1.61
	20.2	98.1	0.22	0.11	0.97	0.42	1.41
SEW-WI-30S-10%	2.2	20.2	ND	0.03	0.27	0.29	0.60
SEW-WI-60S-10%	7.6	86.5	0.24	0.11	2.12	0.44	2.05
Xylan -5%	10.3	0.0	ND	0.03	0.30	0.29	0.23
SEW-WS-15S-10%	10.7	78.5	0.57	0.15	3.83	0.61	2.51
	20.0	55.8	0.82	0.25	6.24	0.92	3.37
SEW-WS-30S-10%	11.3	76.1	0.52	0.16	4.62	0.62	3.26
	20.3	69.5	0.69	0.21	6.33	0.65	3.60
SEW-WS-60S-10%	9.8	75.8	0.32	0.14	3.69	0.52	3.04
	19.5	52.8	0.35	0.13	5.13	0.47	3.31

[a] Cultures were grown anaerobically (H_2) on wood substrate hydrolyzates (Table 17) supplemented with CAB medium. Incubation was carried out statically at 37°C.

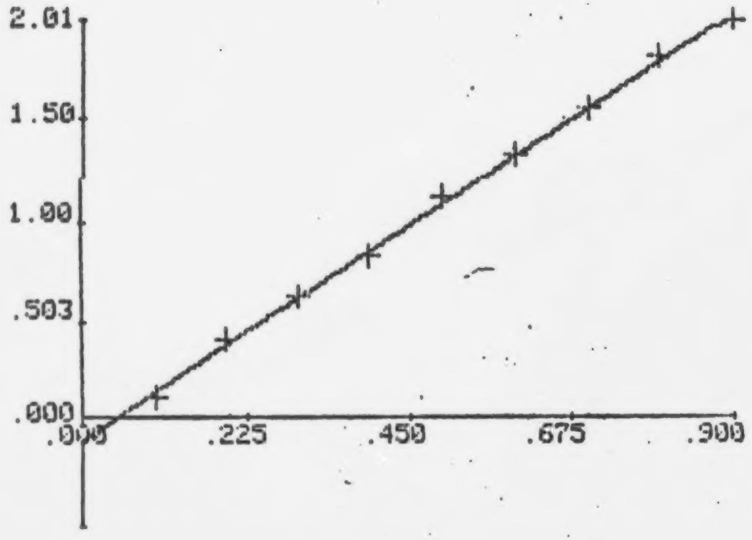
[b] Not detected.

Fig. 1 Standard curves for reducing sugar analysis by DNS

```
GLUCOSE

              CALIBRATION
STNDRD  CONCEN-  CALC.  ABSORB- PERCENT
  #     TRATION   CON.    ANCE    ERROR
---------------------------------------
 1        .1     .09     .095    -9.2
 2        .2     .207    .375    3.8
 3        .3     .303    .604    1.1
 4        .4     .388    .809    -2.8
 5        .5     .512    1.104   2.4
 6        .6     .6      1.316   0
 7        .7     .699    1.554   -.1
 8        .8     .805    1.807   .6
 9        .9     .891    2.013   -1

BEST FIT SLOPE = 2.39583333
BEST FIT INTERCEPT = -.122694443
R**2 = .99912471
```



```
XYLOSE

              CALIBRATION
STNDRD  CONCEN-  CALC.  ABSORB- PERCENT
  #     TRATION   CON.    ANCE    ERROR
---------------------------------------
 1        .1     .097    .165    -2.6
 2        .2     .203    .443    1.9
 3        .3     .295    .681    -1.7
 4        .4     .4      .956    .1
 5        .5     .505    1.23    1.1
 6        .6     .607    1.496   1.2
 7        .7     .687    1.706   -1.8
 8        .8     .798    1.995   -.2
 9        .9     .903    2.268   .3

BEST FIT SLOPE = 2.60366667
BEST FIT INTERCEPT = -.0892777786
R**2 = .99951613
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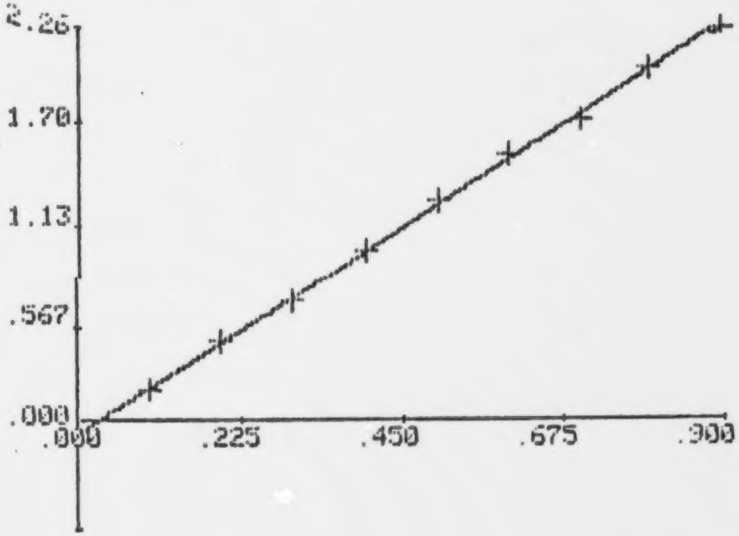


Fig. 2 Chromatogram of HPLC analysis of sugars

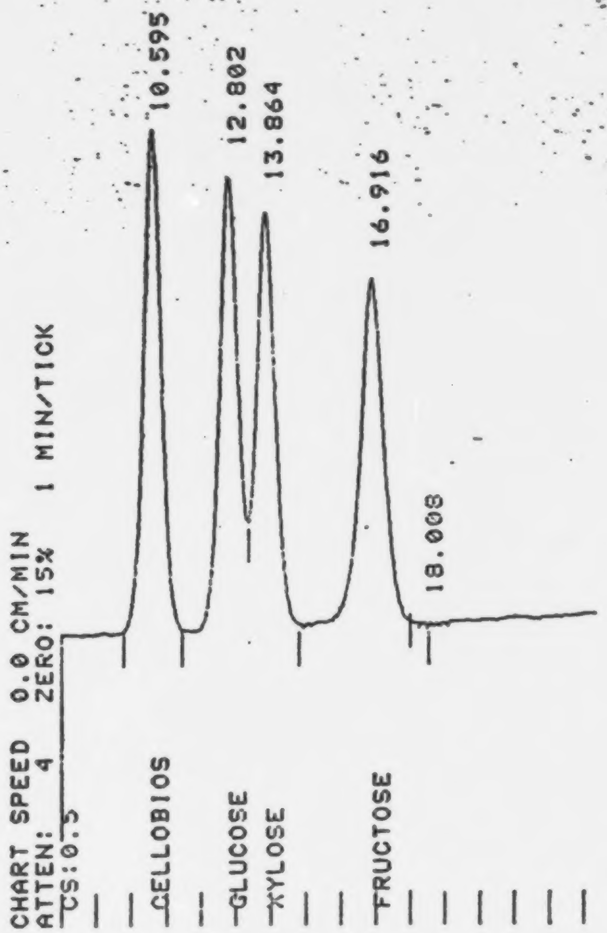
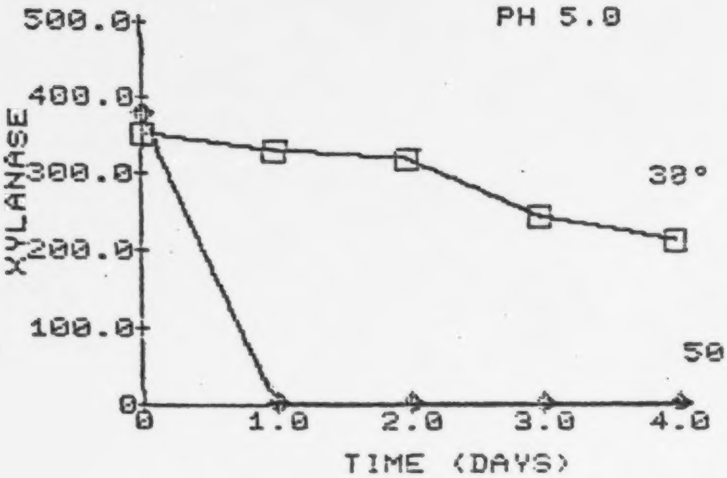
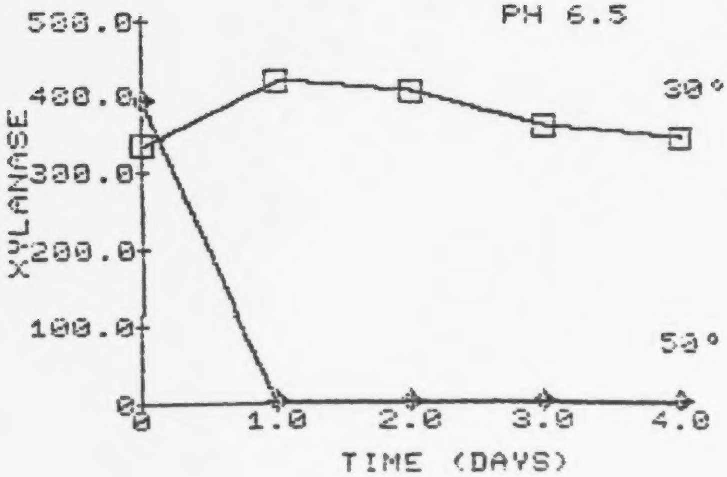


Fig. 3 Stability of xylanase enzymes under various hydrolysis conditions





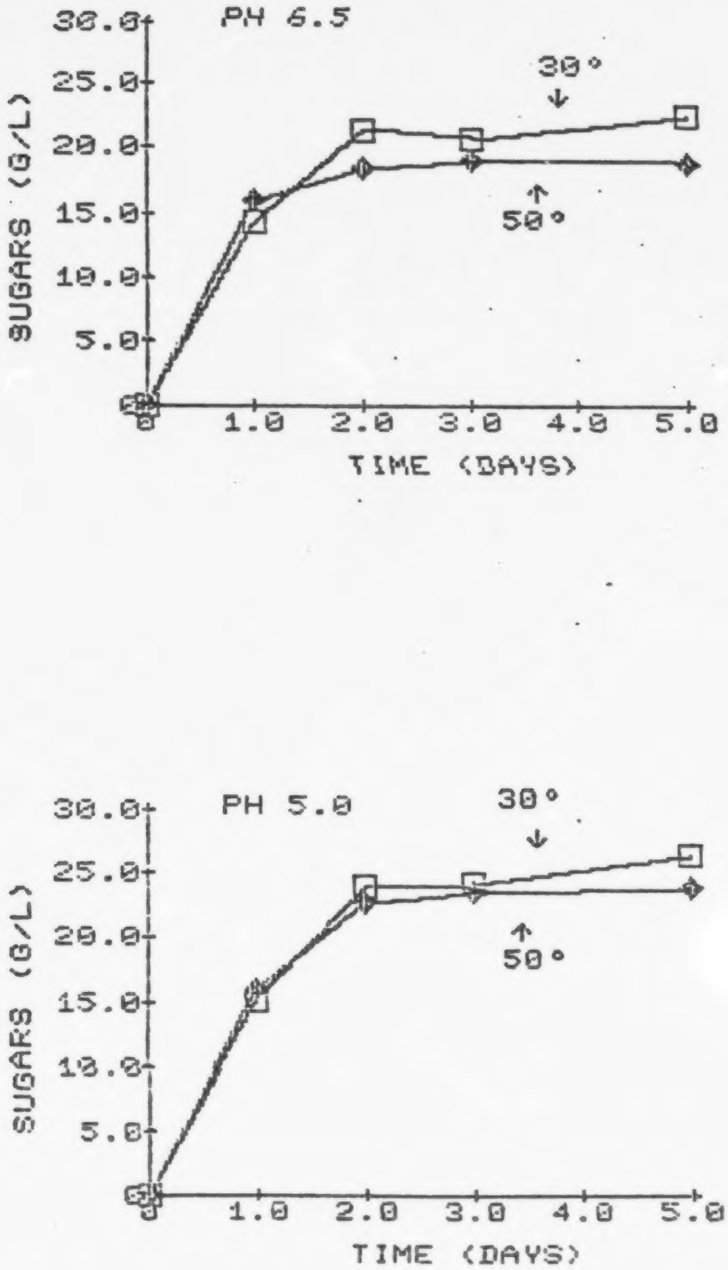


Fig. 4 Release of reducing sugars during hydrolysis of xylan by *T. harzianum* E58 xylanase enzymes

Fig. 5 Release of sugars during hydrolysis of xylan by *T*. harzianum E58 enzymes at 30°C, pH 5.0

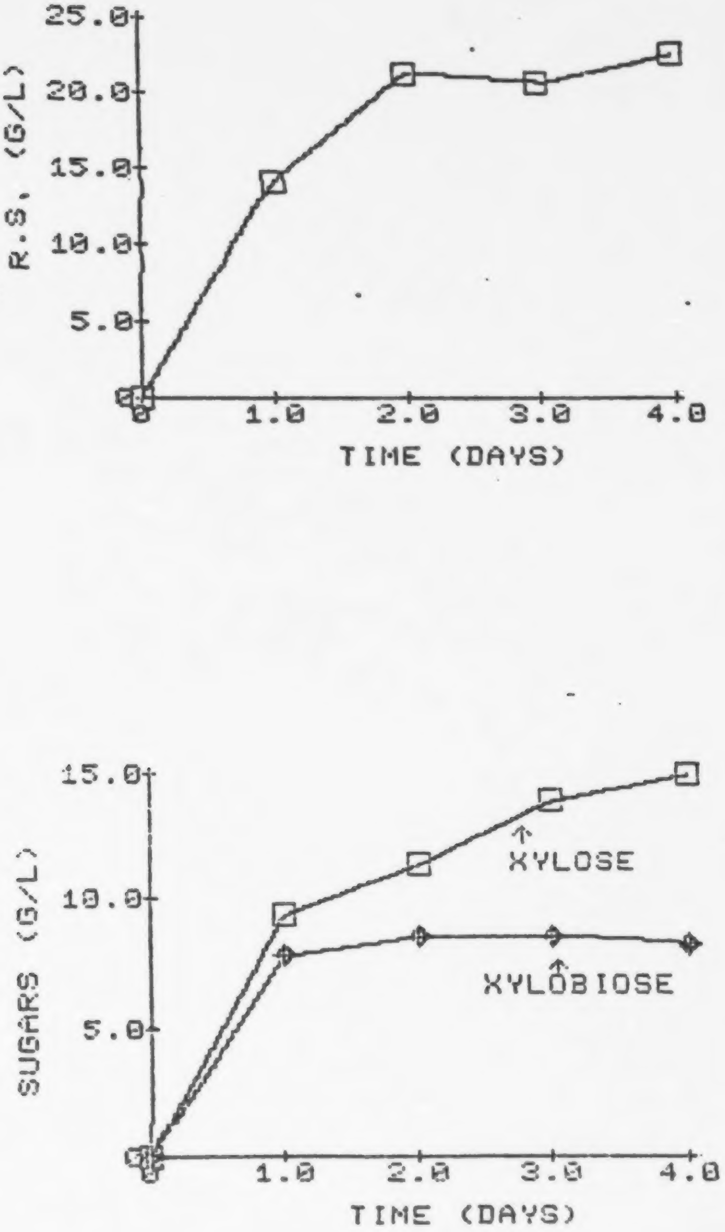


Fig. 6 Gas chromatographic analysis of volatile products in butanediol fermentation

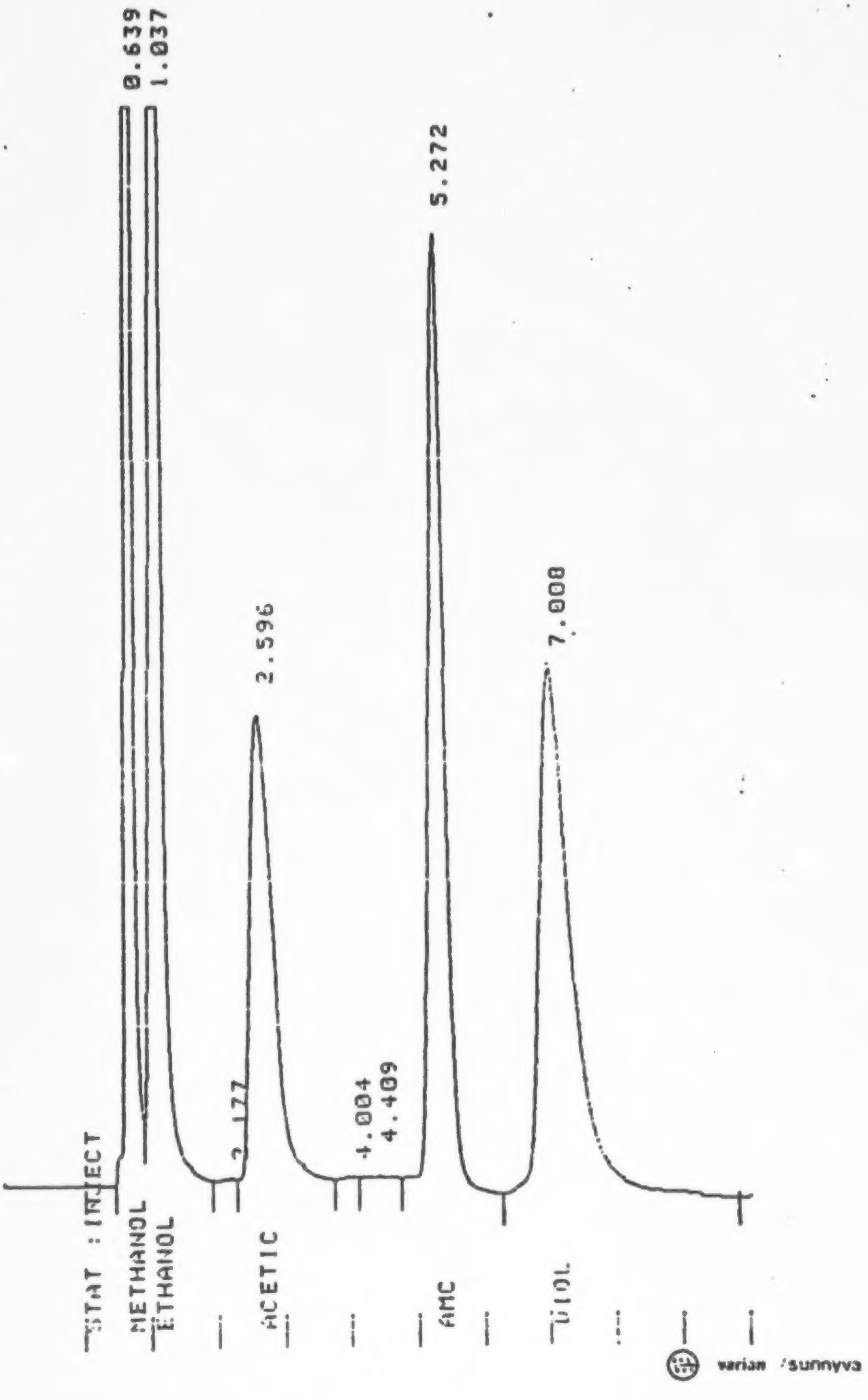


Fig. 7 Effect of added acetic acid on butanediol fermentation of *K. pneumoniae* grown on D-glucose

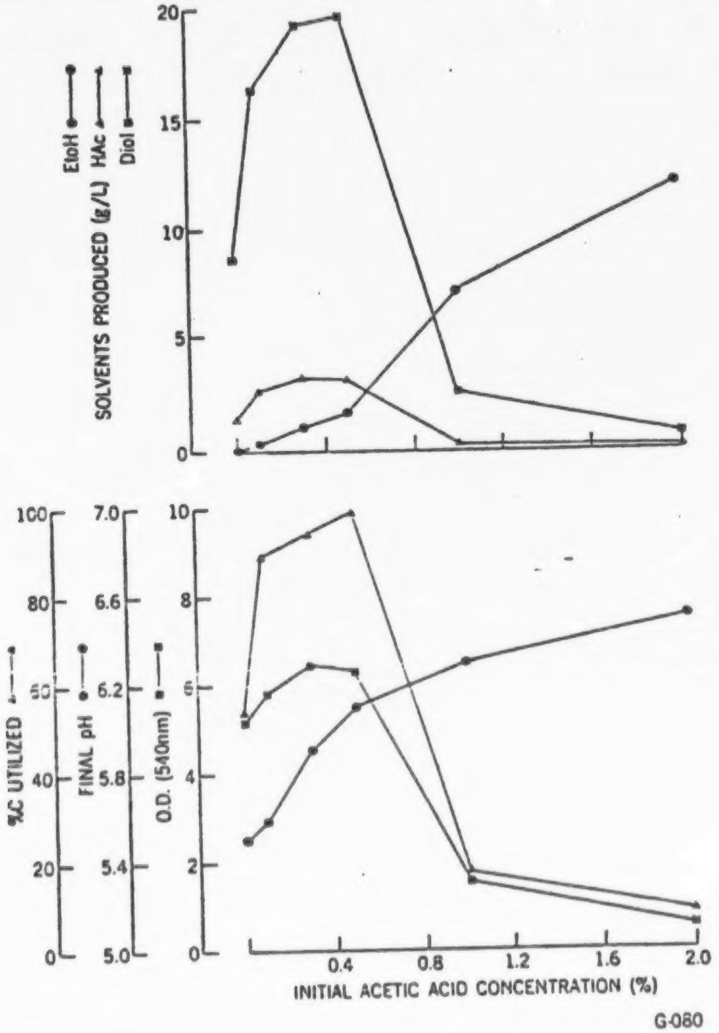


Fig. 8 Effect of acetic acid on butanediol production by xylose-grown cells of *K. pneumoniae*

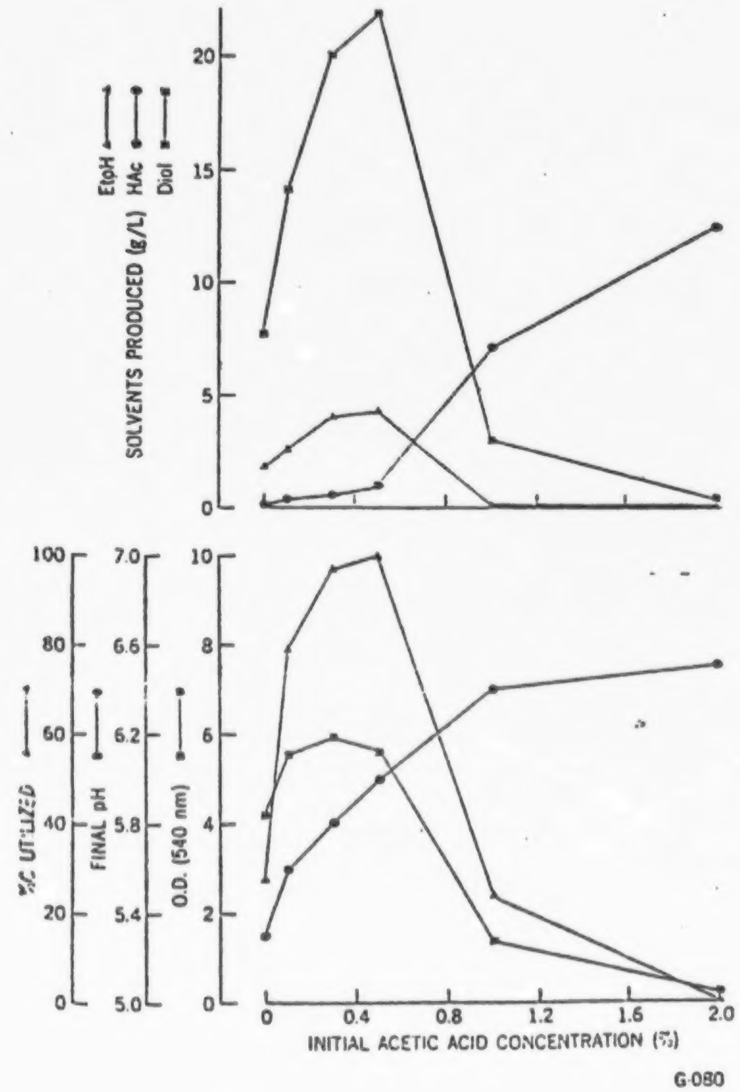


Fig. 9 Effect of the timing of acetic acid addition on butanediol production by *K. pneumoniae*

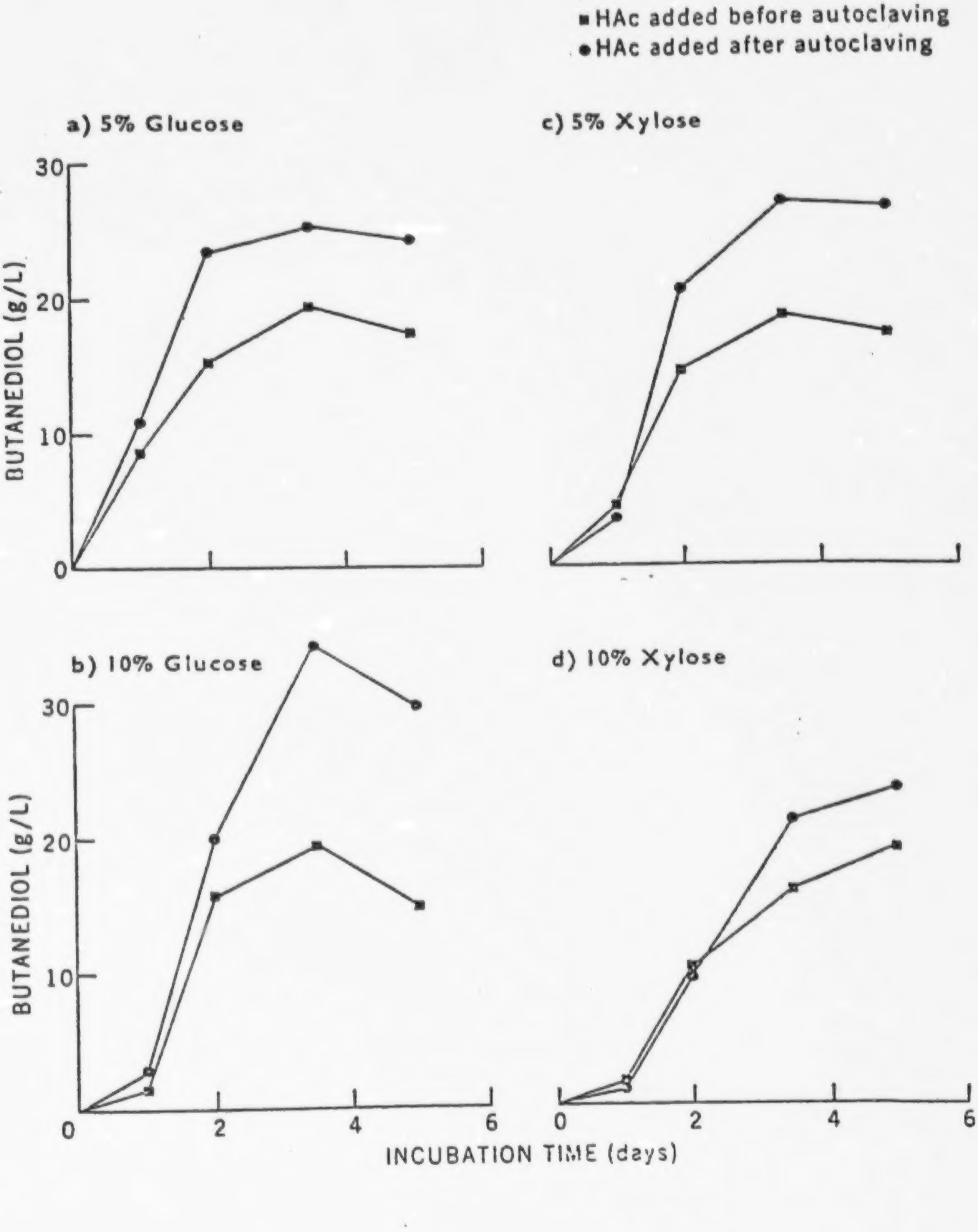


Fig. 10. The effect of initial sugar concentrations on butanediol production by _K_. _pneumoniae_ under finite air conditions.

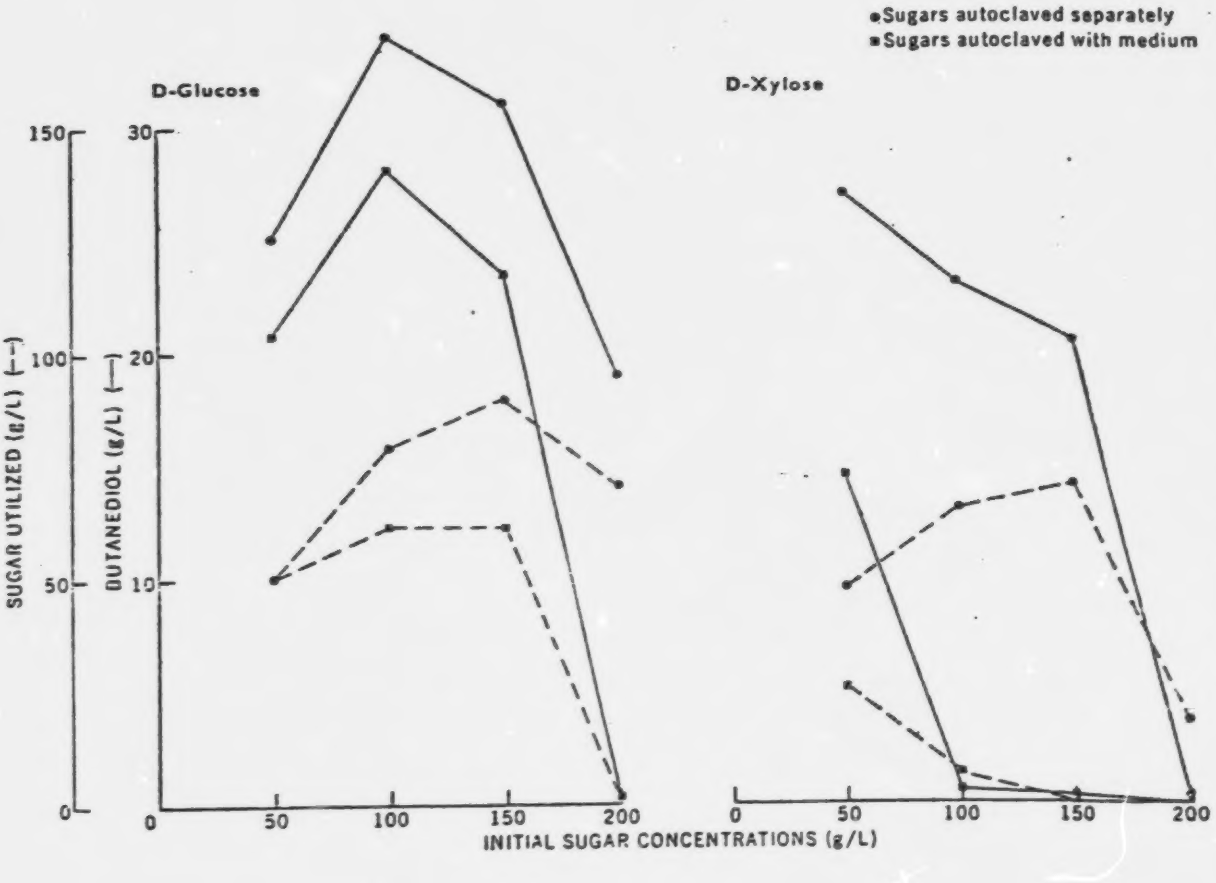


Fig. 11 The influence of initial sugar levels on butanediol production and sugar utilization by K. pneumoniae under finite air conditions

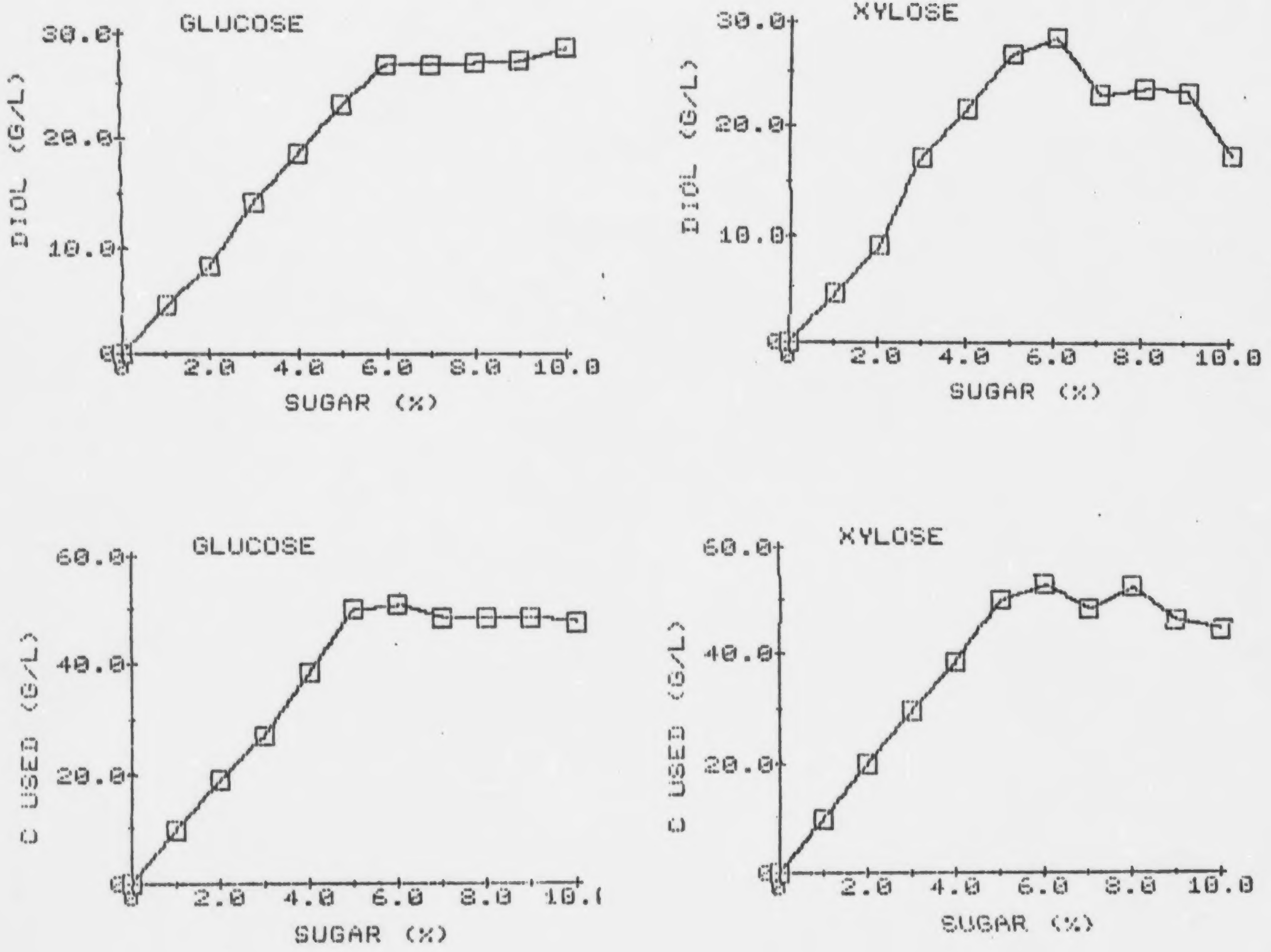


Fig. 12 Simple fed-batch system for butanediol fermentation of *K. pneumoniae* grown on glucose under finite air conditions

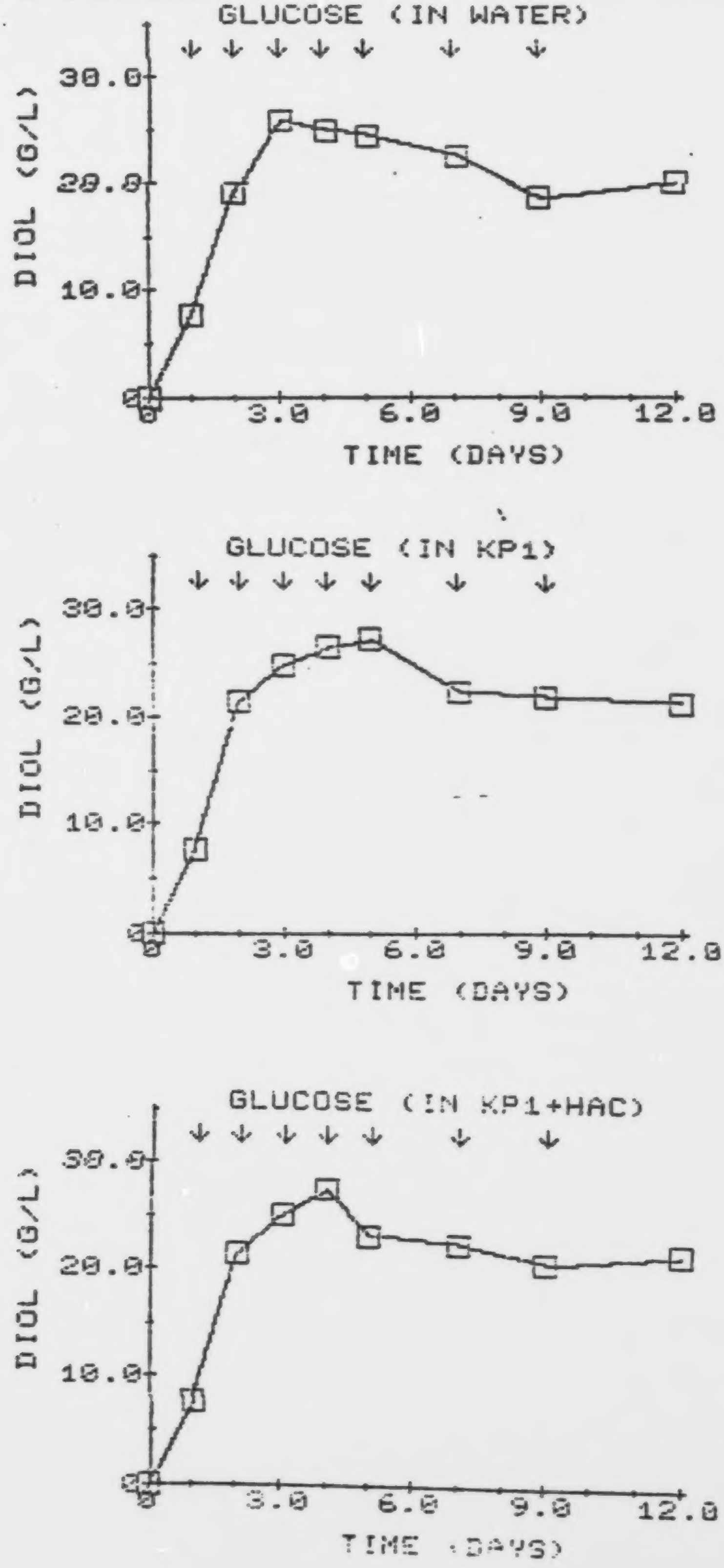


Fig. 13 Fed-batch system of xylose-grown *K. pneumoniae* cells for butanediol production under finite air conditions

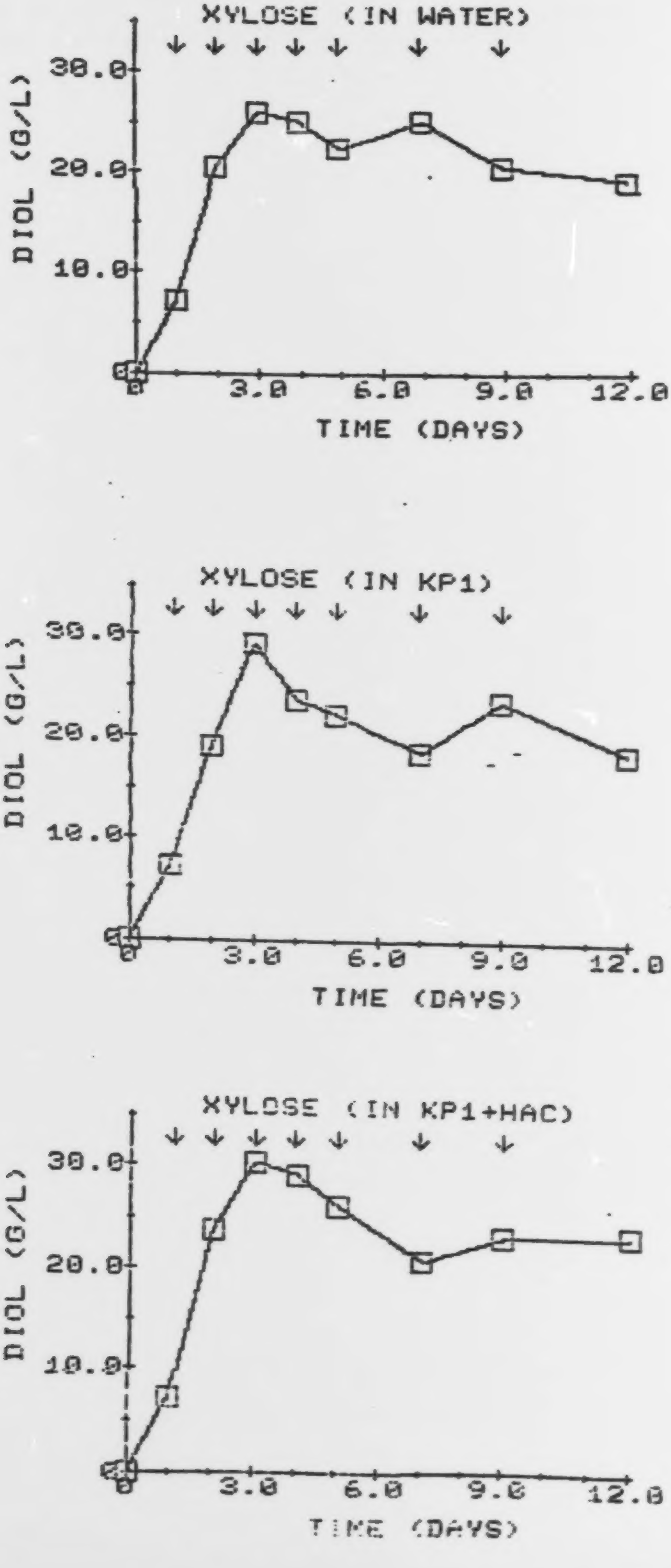


Fig. 14 Butanediol production of *K. pneumoniae* grown on D-xylose under finite air conditions using a fed-batch approach

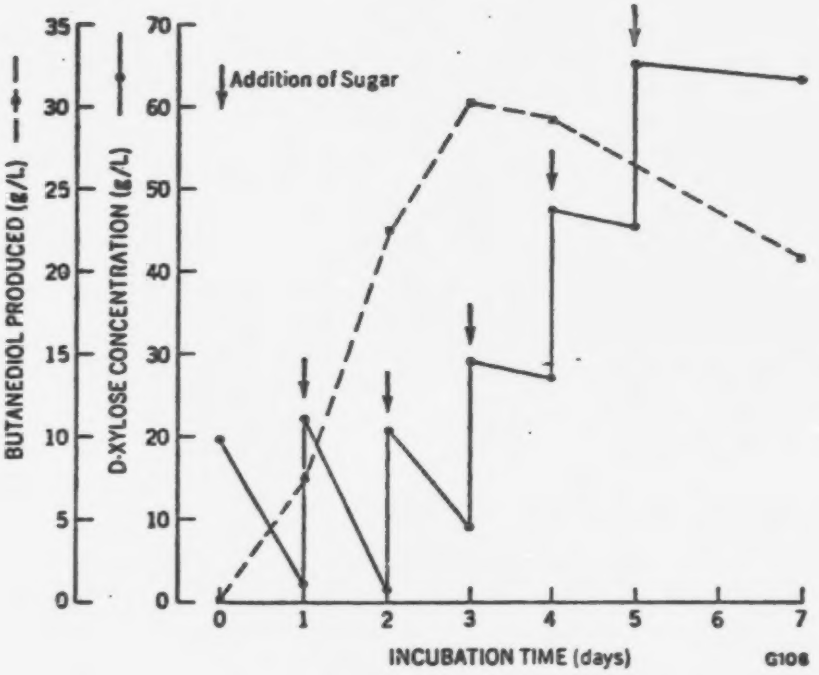
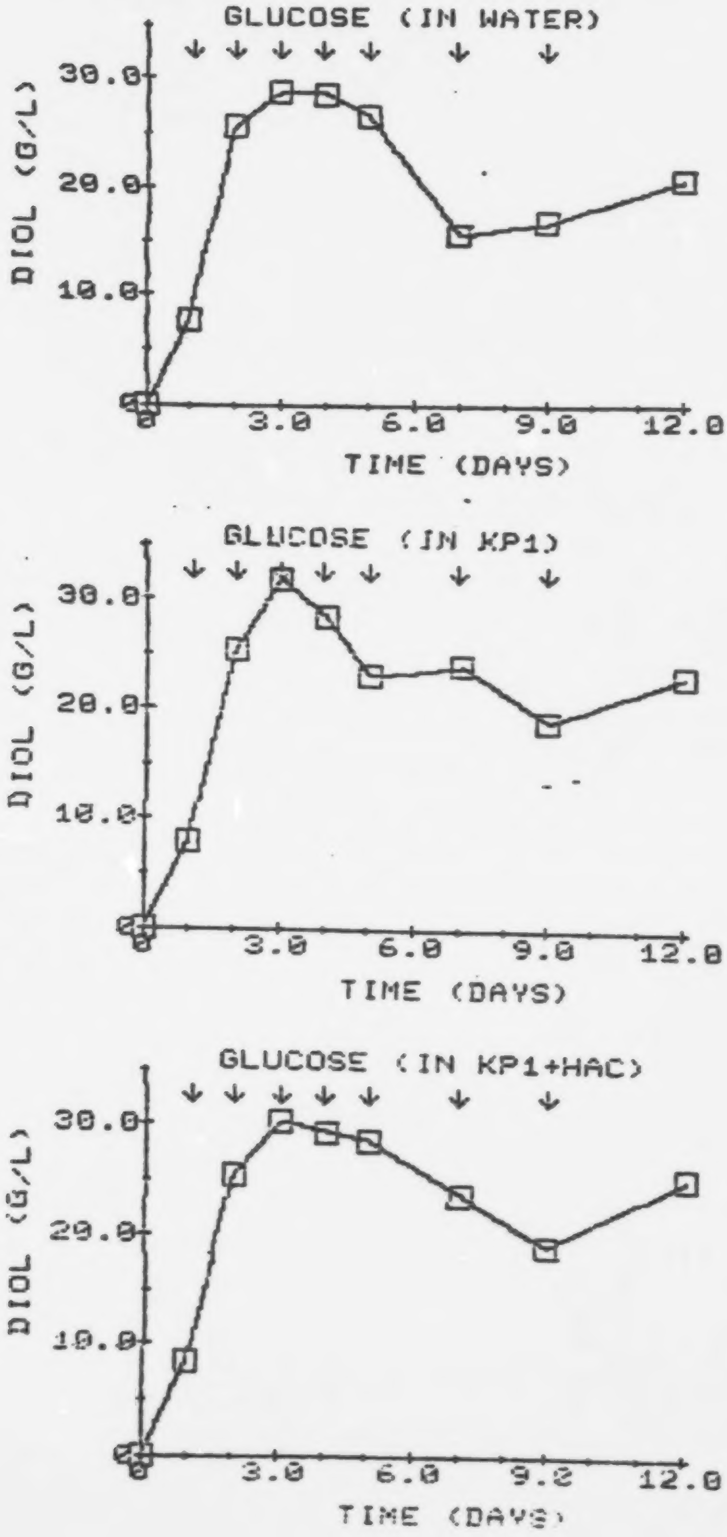


Fig. 15 Semi-continuous fed-batch approach to butanediol production by *K. pneumoniae* grown on glucose under finite air conditions



by K. pneumoniae grown on xylose under finite air conditions

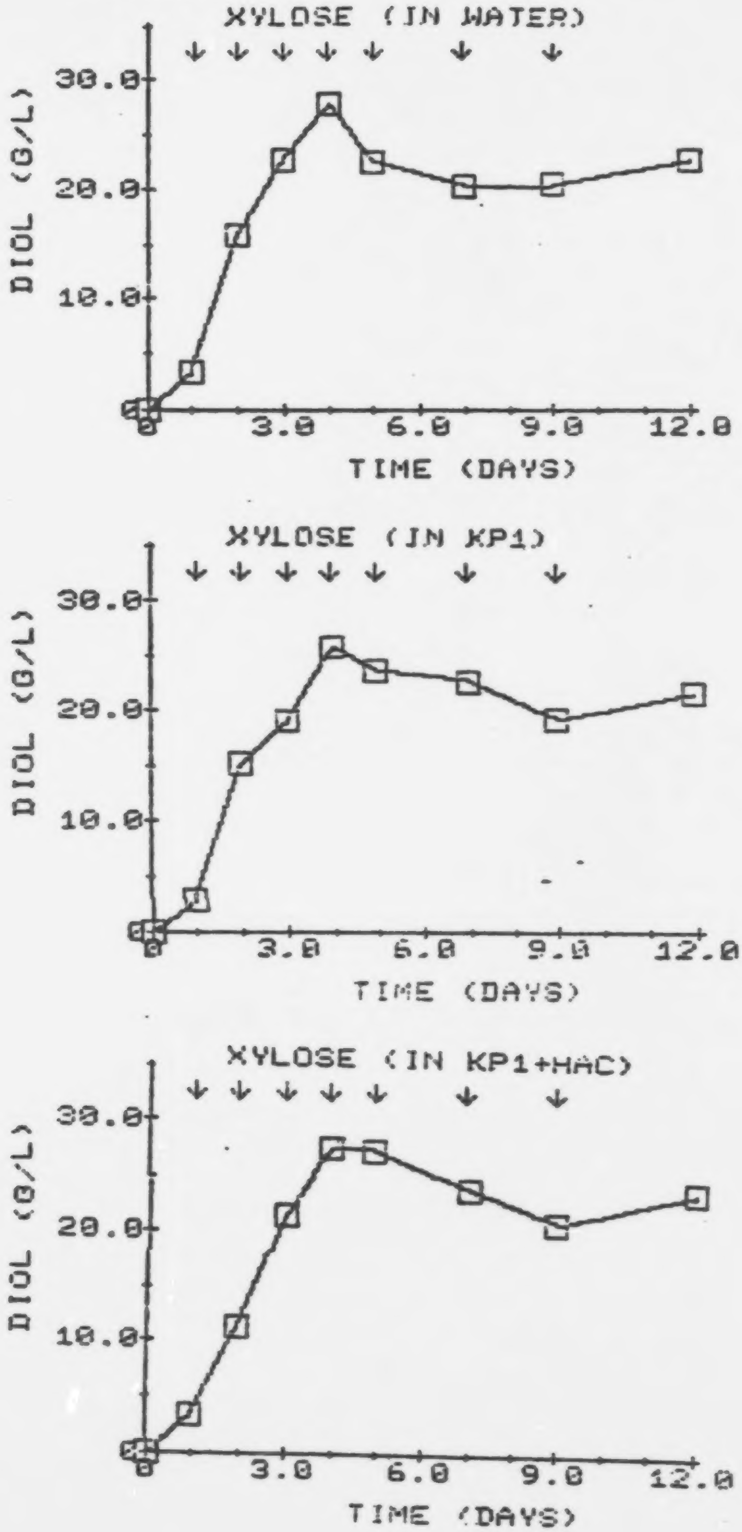


Fig. 17 Double fed-batch approach to butanediol production and sugar utilization by K. pneumoniae grown under aerobic (☐) and finite air (▦) conditions

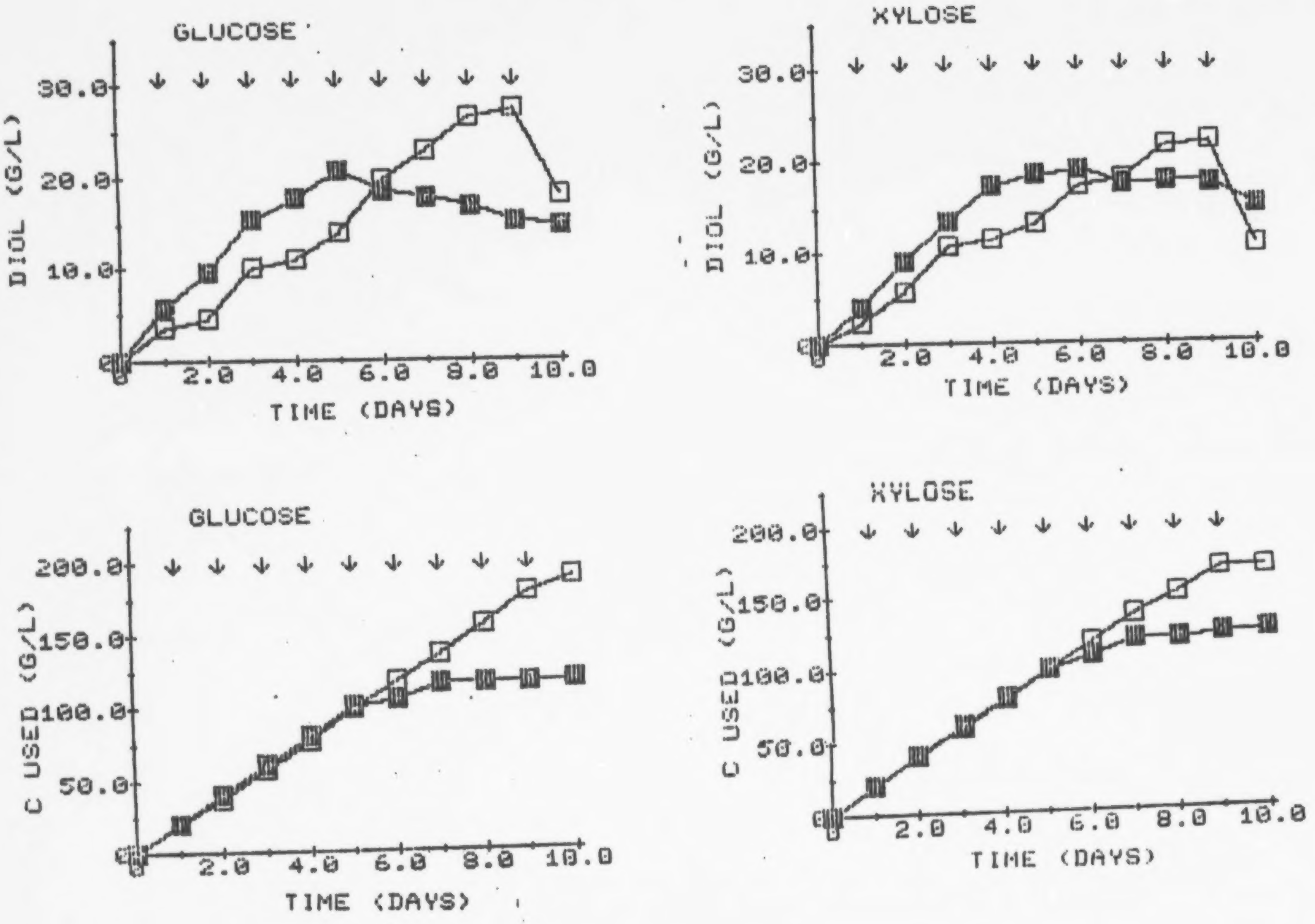
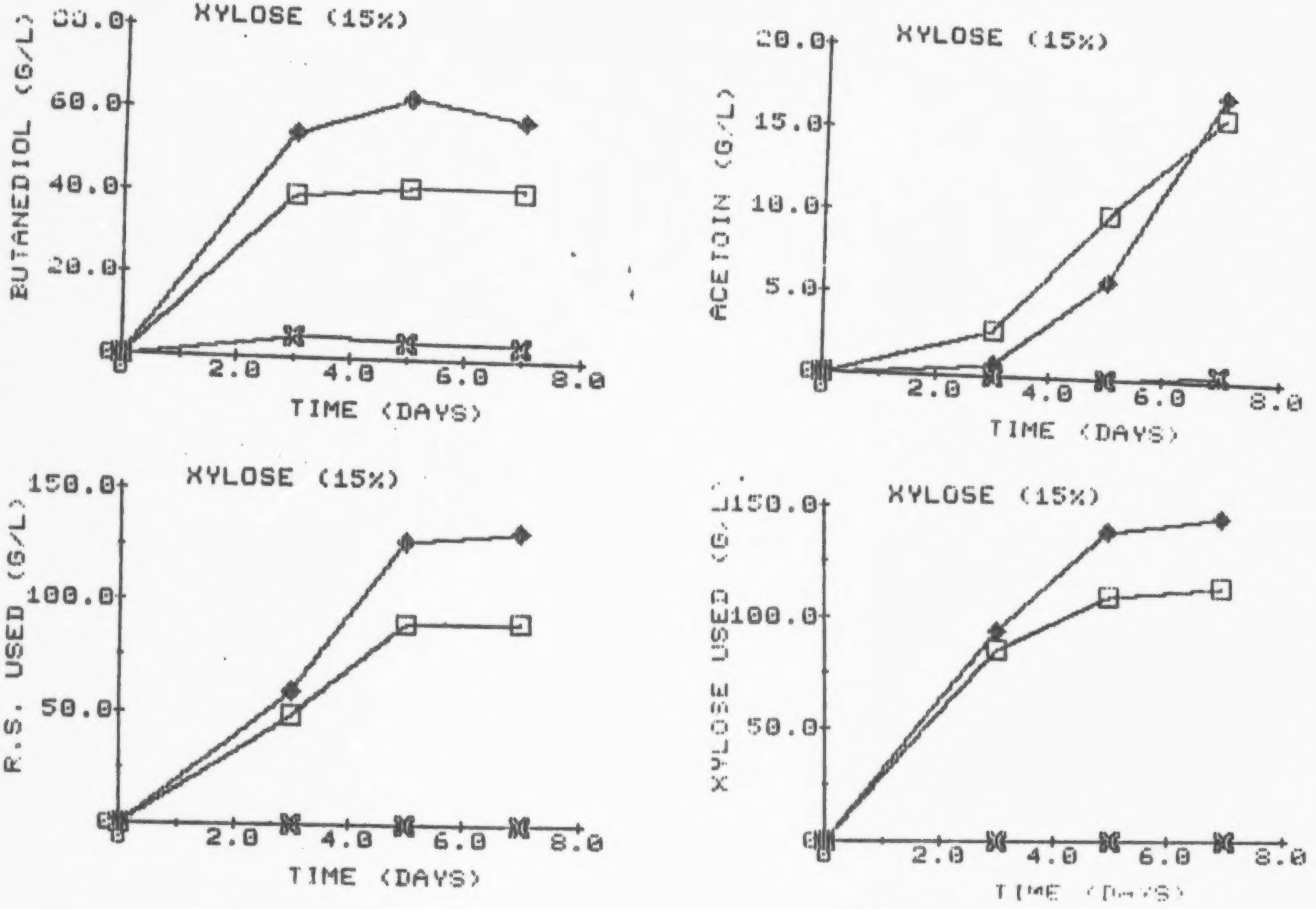


Fig. 18 The effect of medium modification on butanediol fermentation of K. pneumoniae grown aerobically using acclimatized cultures. ◆, YE + HAc; □, YE; ⋈, HAc alone.



grown aerobically at initial glucose level of 10% or 15%. Solvents: ☐ Butanediol, ◆ Acetoin; C used: ☐ Reducing sugars, ◆ Monosaccharides.

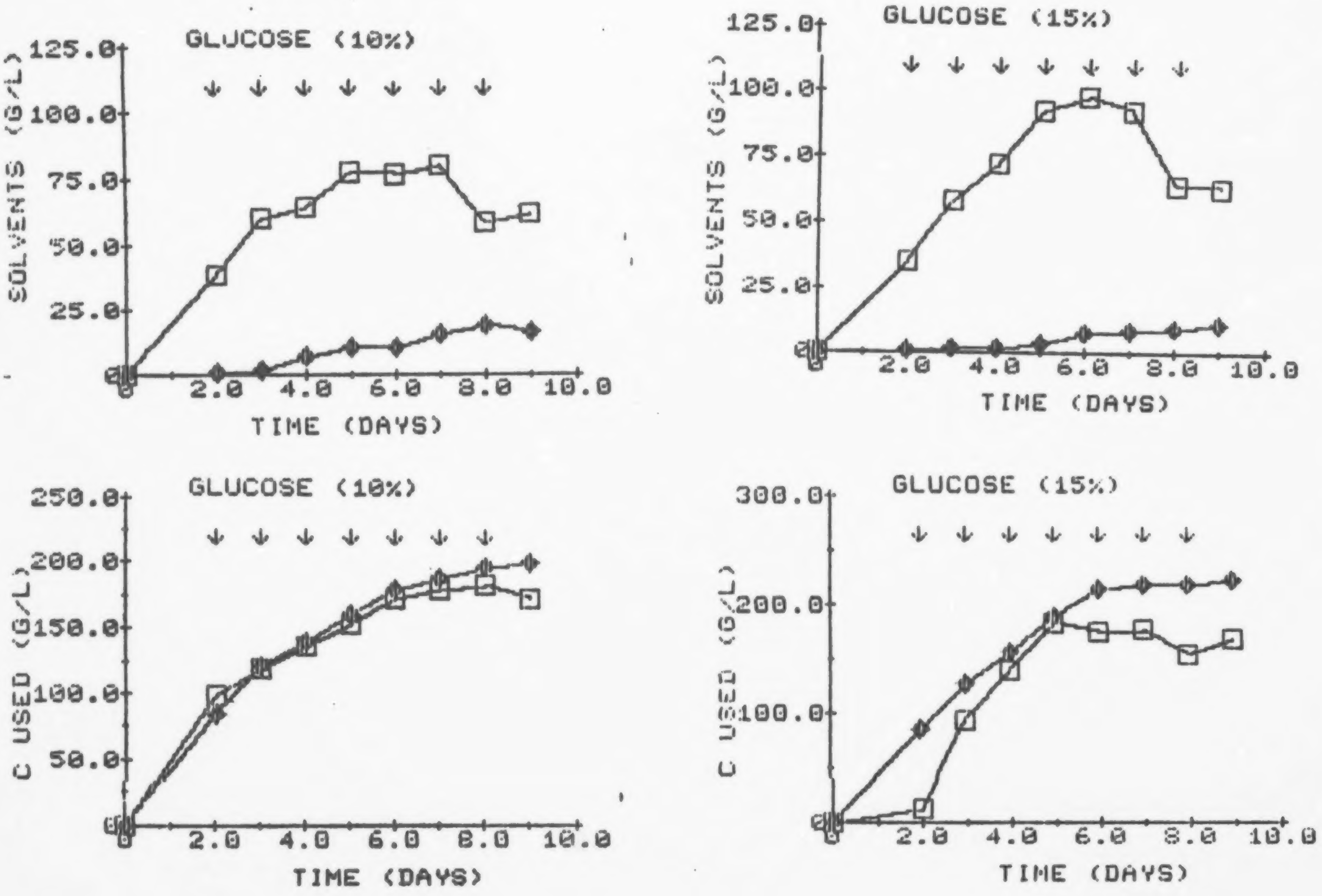
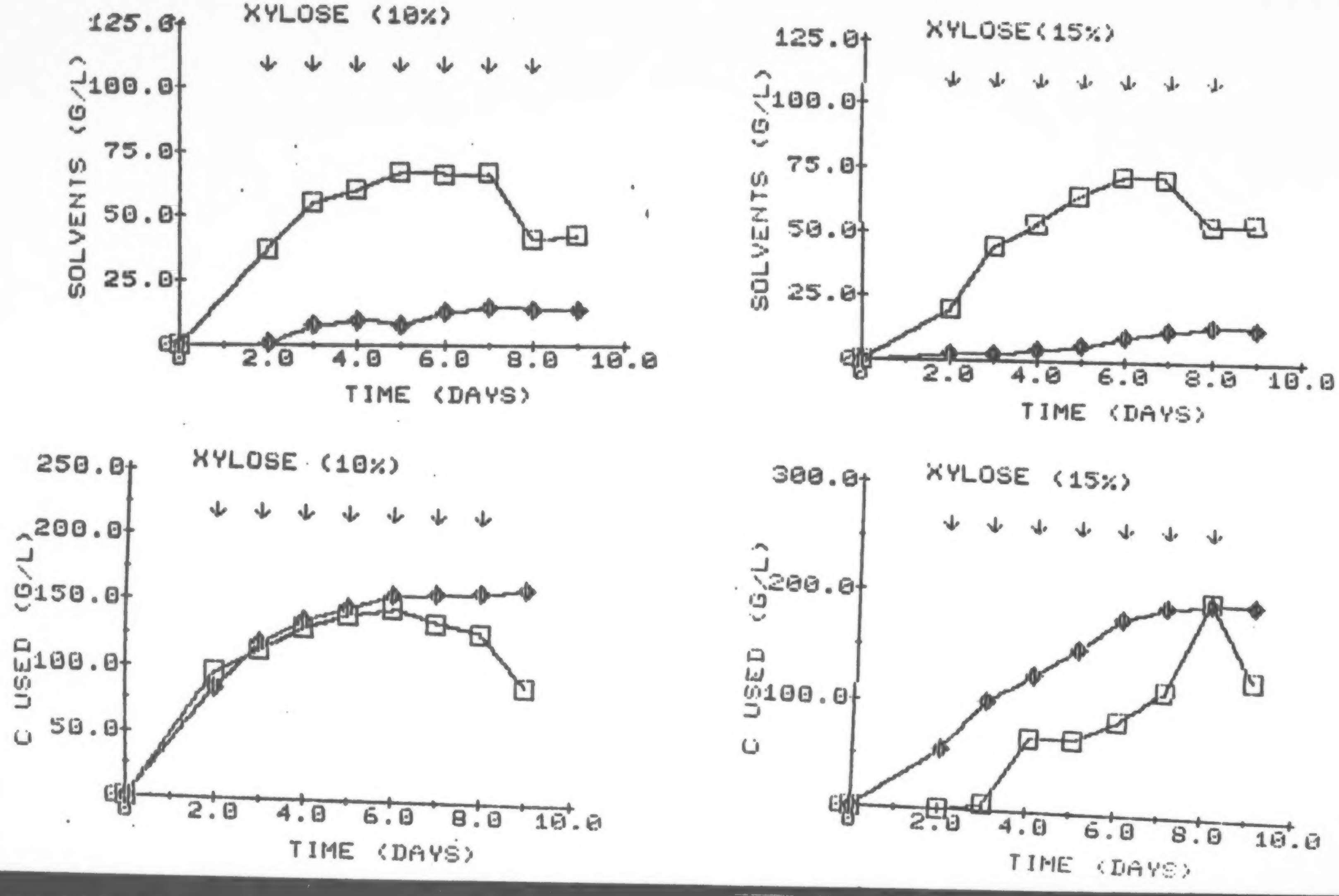


Fig. 20 Fed-batch approach to butanediol fermentation by K. pneumoniae acclimatized to xylose. Please see Fig. 19 for legends



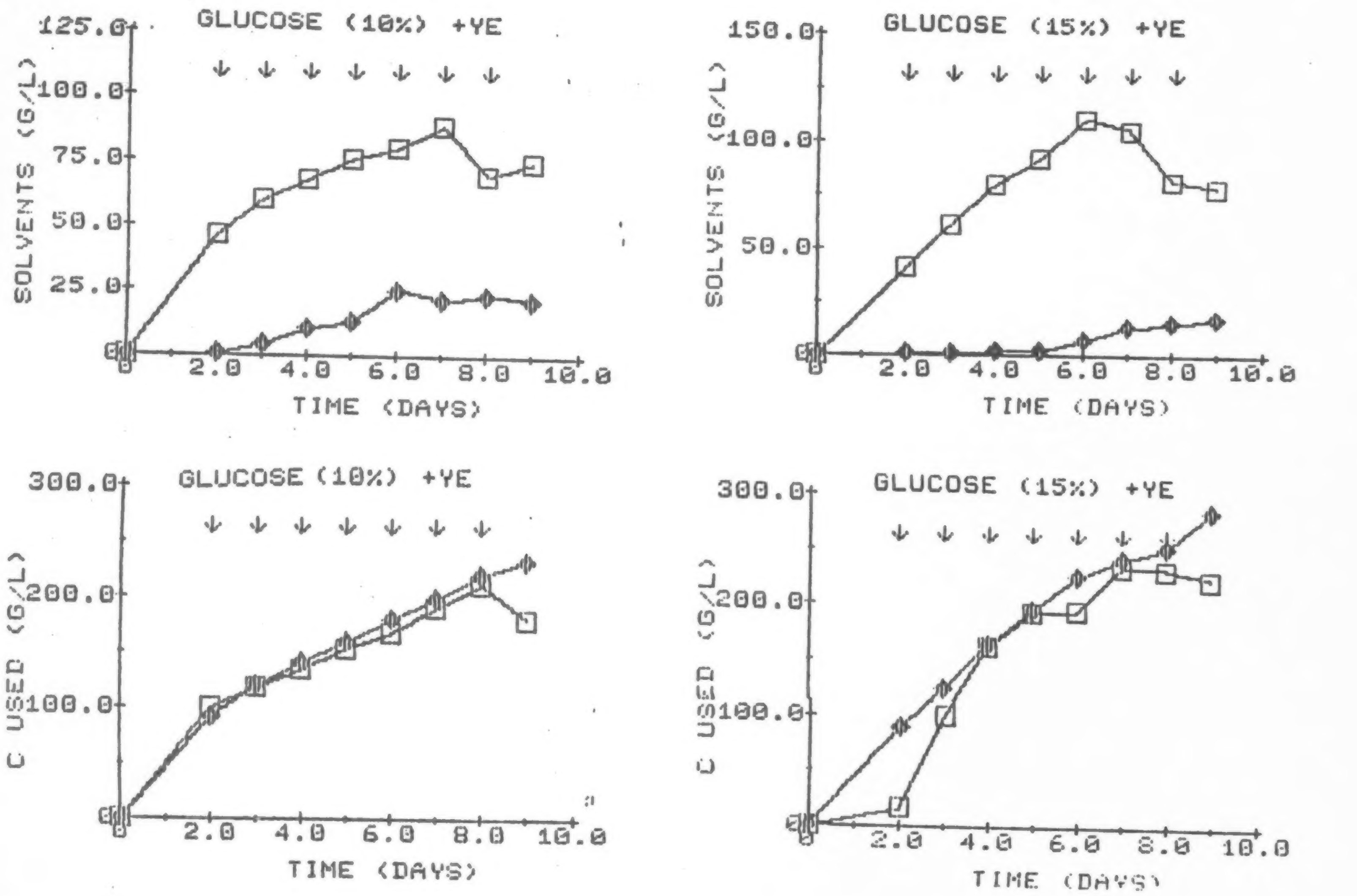


Fig. 21 Double fed-batch approach to butanediol fermentation by acclimatized *K. pneumoniae* cells. Refer to Fig. 19 for legends

Fig. 22 Double fed-batch approach to butanediol fermentation by _K. pneumoniae_ acclimatized cells to high xylose concentrations. Refer to Fig. 19 for legends

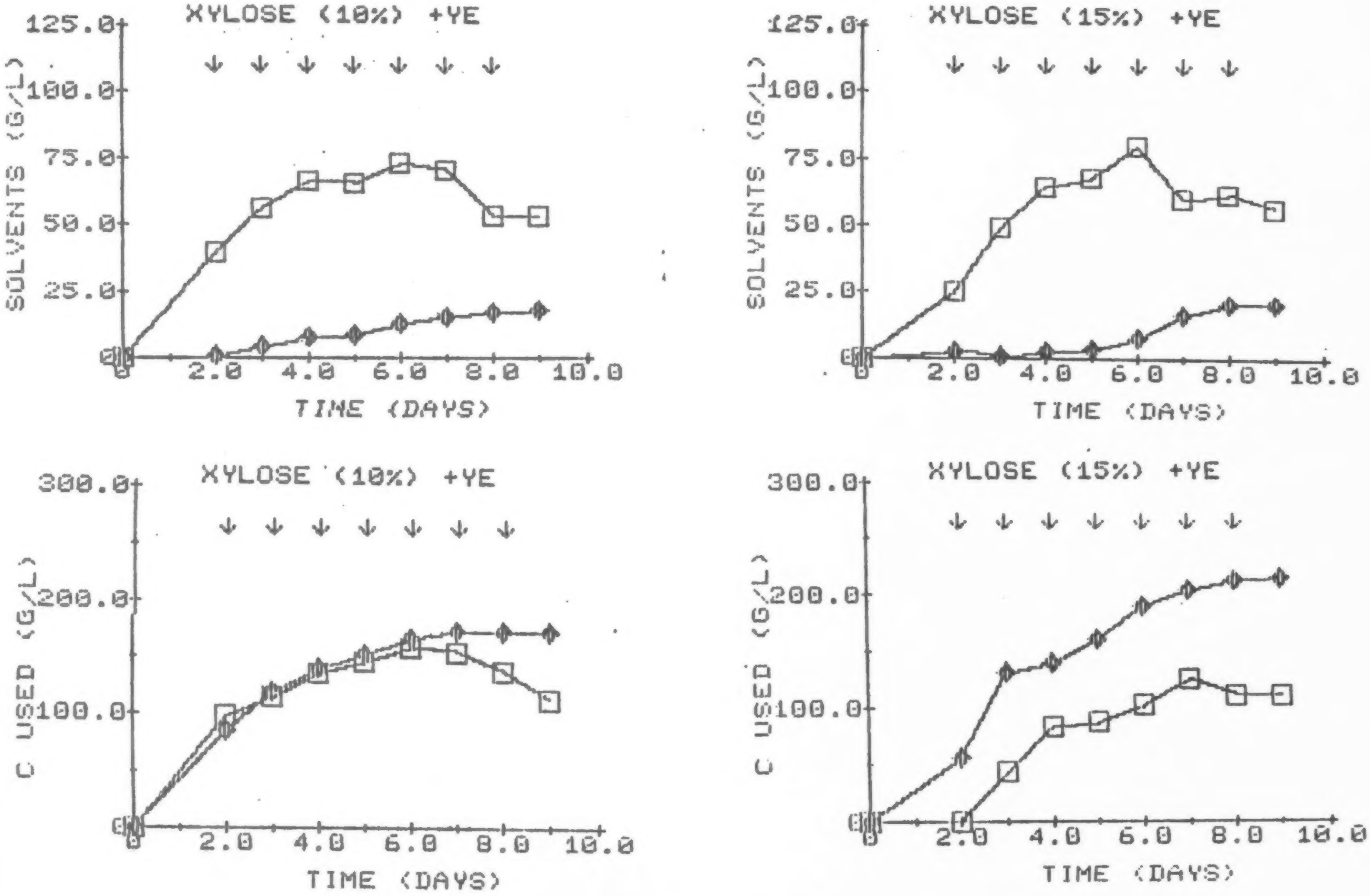


Fig. 23 Butanediol production and sugar utilization by *K. pneumoniae* grown in Kp2 medium. ◆, medium supplemented with *T.* E58 culture filtrate; ☐, control.

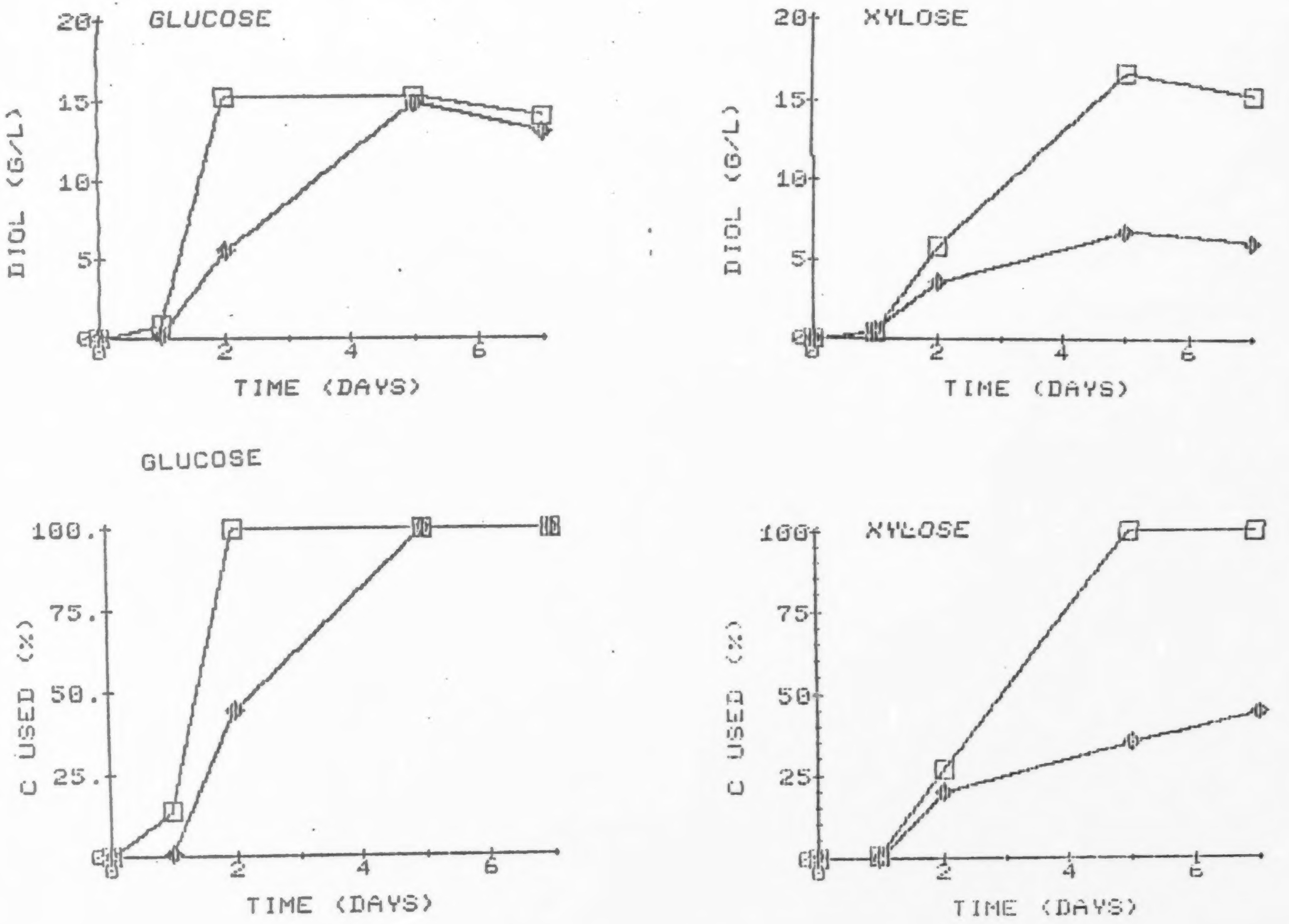


Fig. 24 Fermentor run on butanediol production by *K. pneumoniae* grown in 1.0 L of Kp 2 medium with flushing (0.5 L/min of air). □, butanediol; ◆, acetoin.

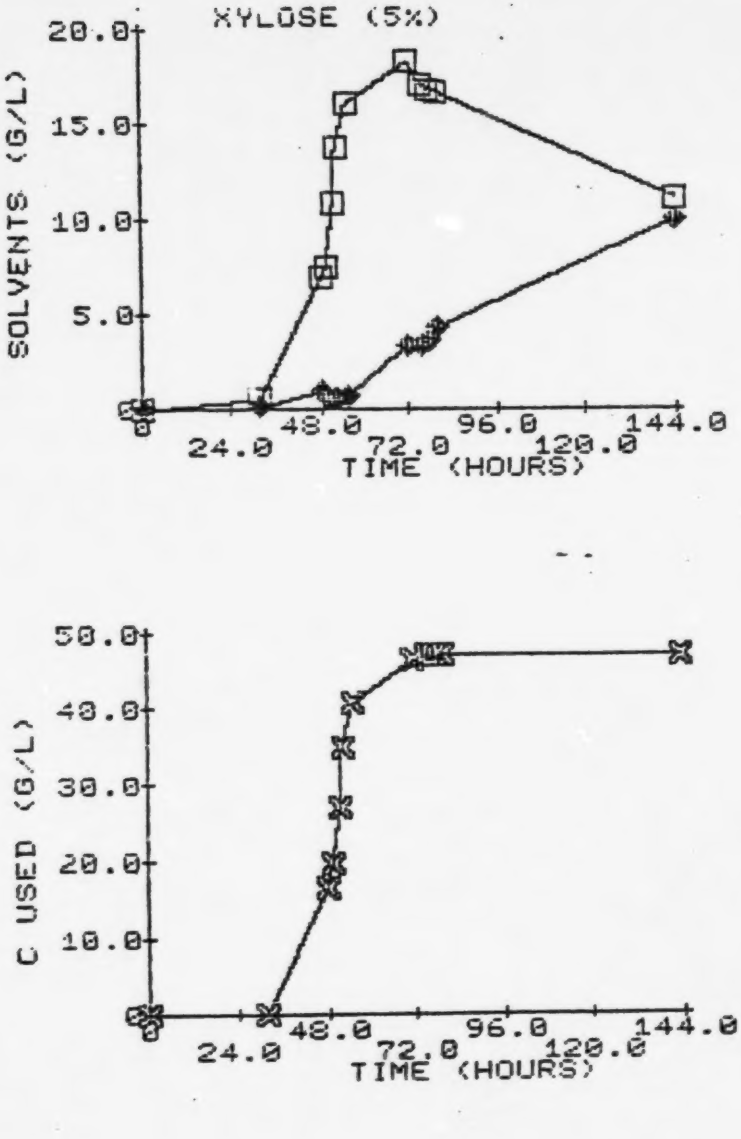


Fig. 25 Fermentor run on butanediol production by _K. pneumoniae_ grown in 1.0 L of Kp 2 medium under finite air conditions (no flushing; no exhaust) □, Butanediol, ◆, Acetoin

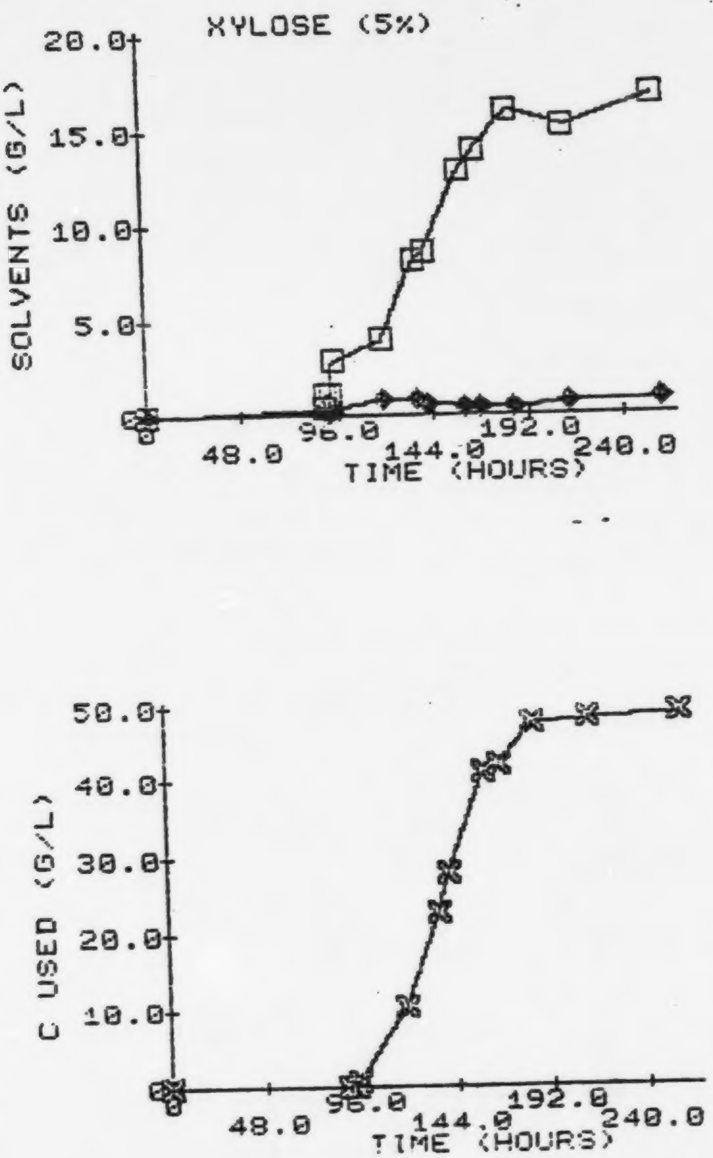


Fig. 26 Fermentor run on butanediol production by *K. pneumoniae* grown in 1.5 L of Kp 2 medium with flushing. □, Butanediol; ◆ acetoin.

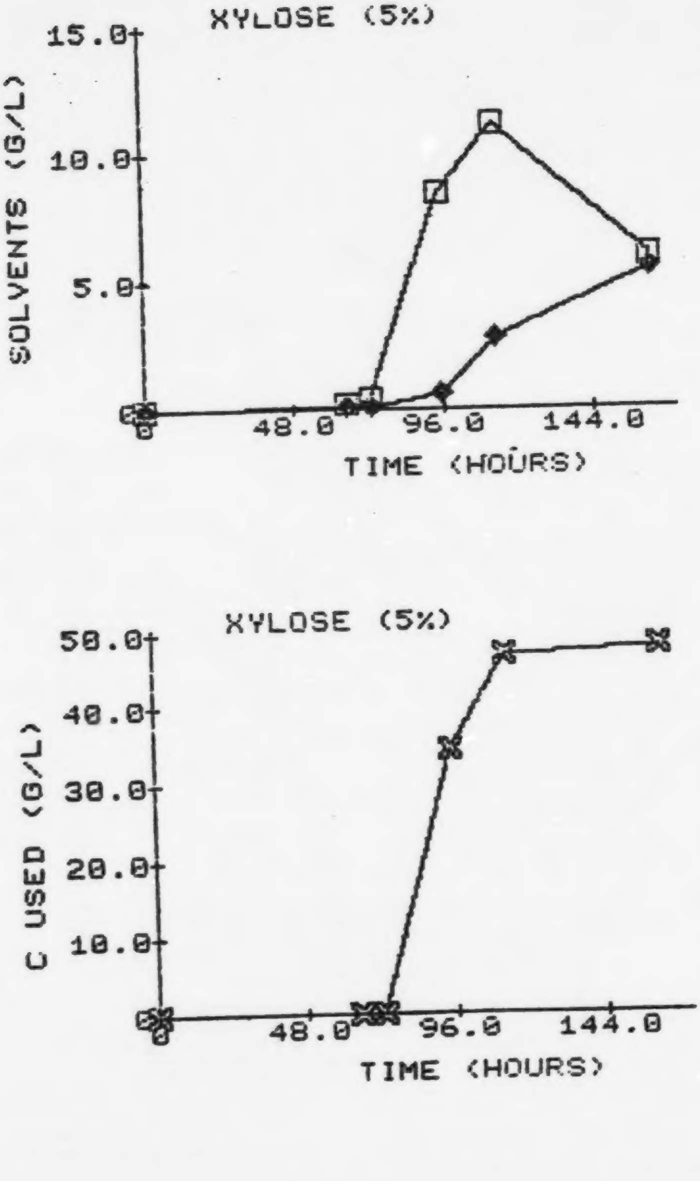


Fig. 27 Fermentor run on butanediol production by *K. pneumoniae* grown in 1.5 L of Kp 2 medium (without MES) with flushing. □ , Butanediol; ◆, Acetoin.

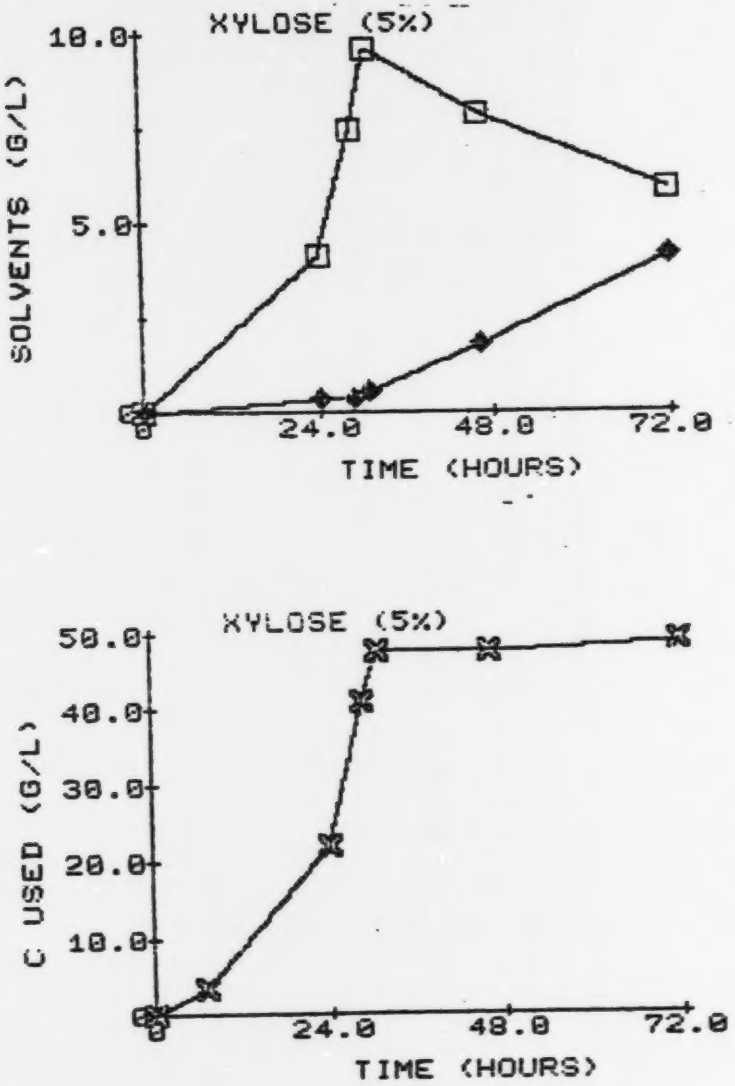


Fig. 28 Gas chromatographic analysis of volatile products of acetone-butanol fermentation by C. acetobutylicum

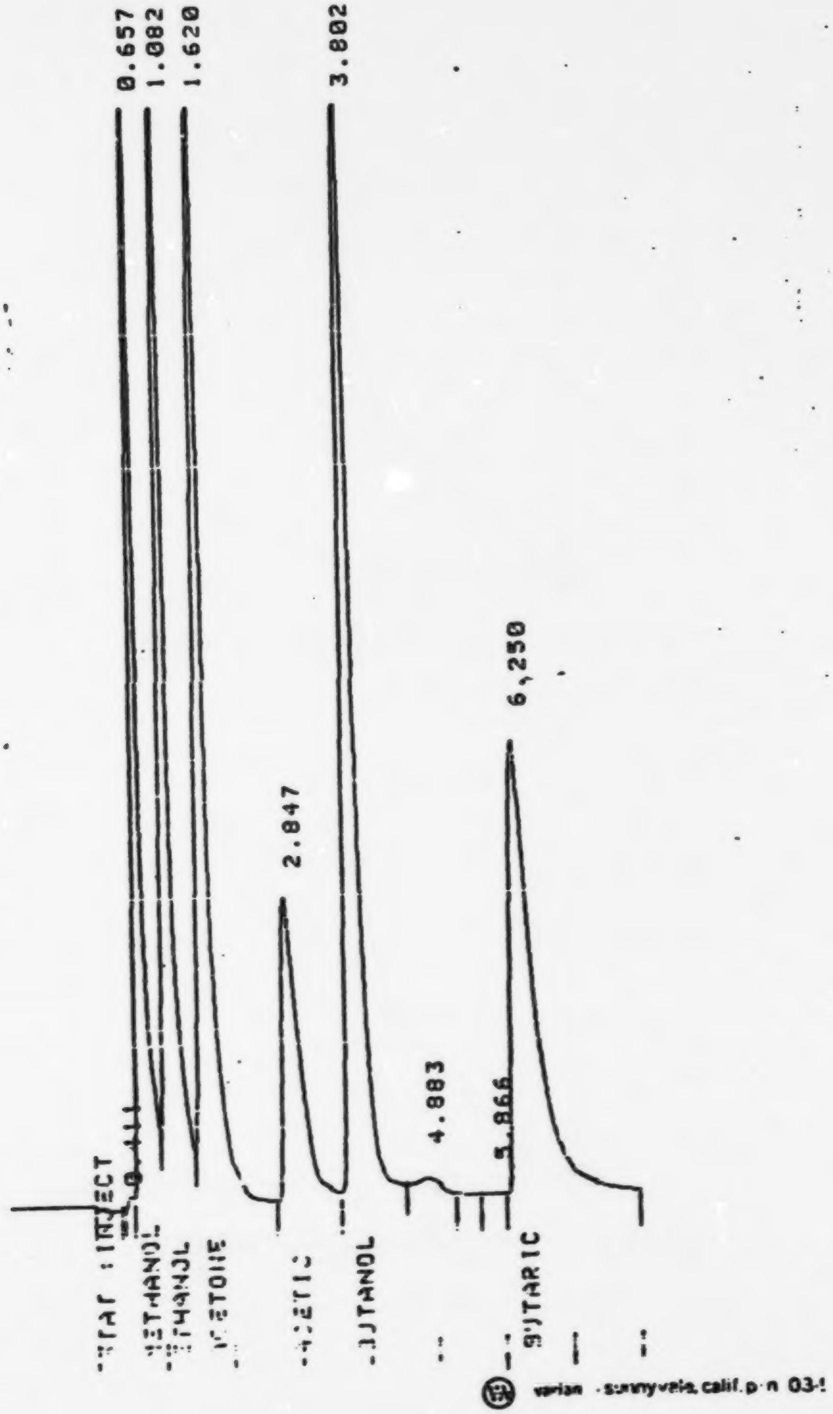


Fig. 29 The effect of medium cysteine-HCl concentration on the solvent production of *C. acetobutylicum*

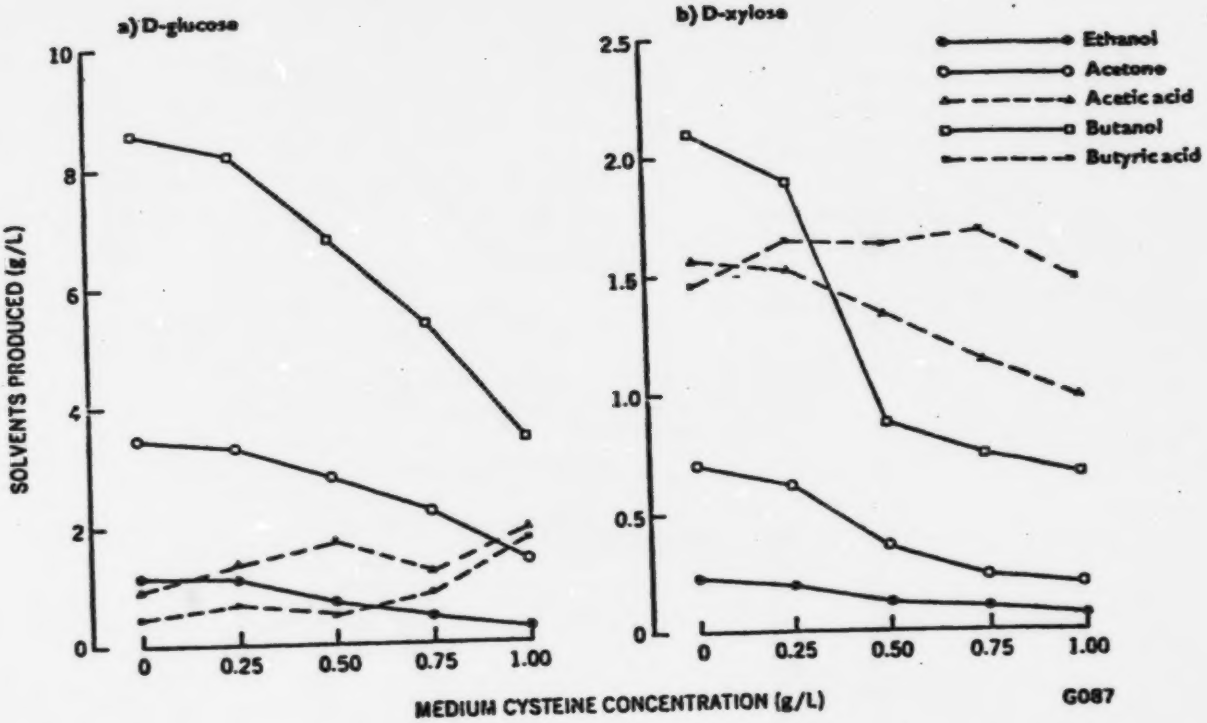


Fig. 30 The influence of initial pH on acetone-butanol fermentation of *C. acetobutylicum* grown on glucose. Solvents: □, butanol; ◆, acetone; ⌘, ethanol.

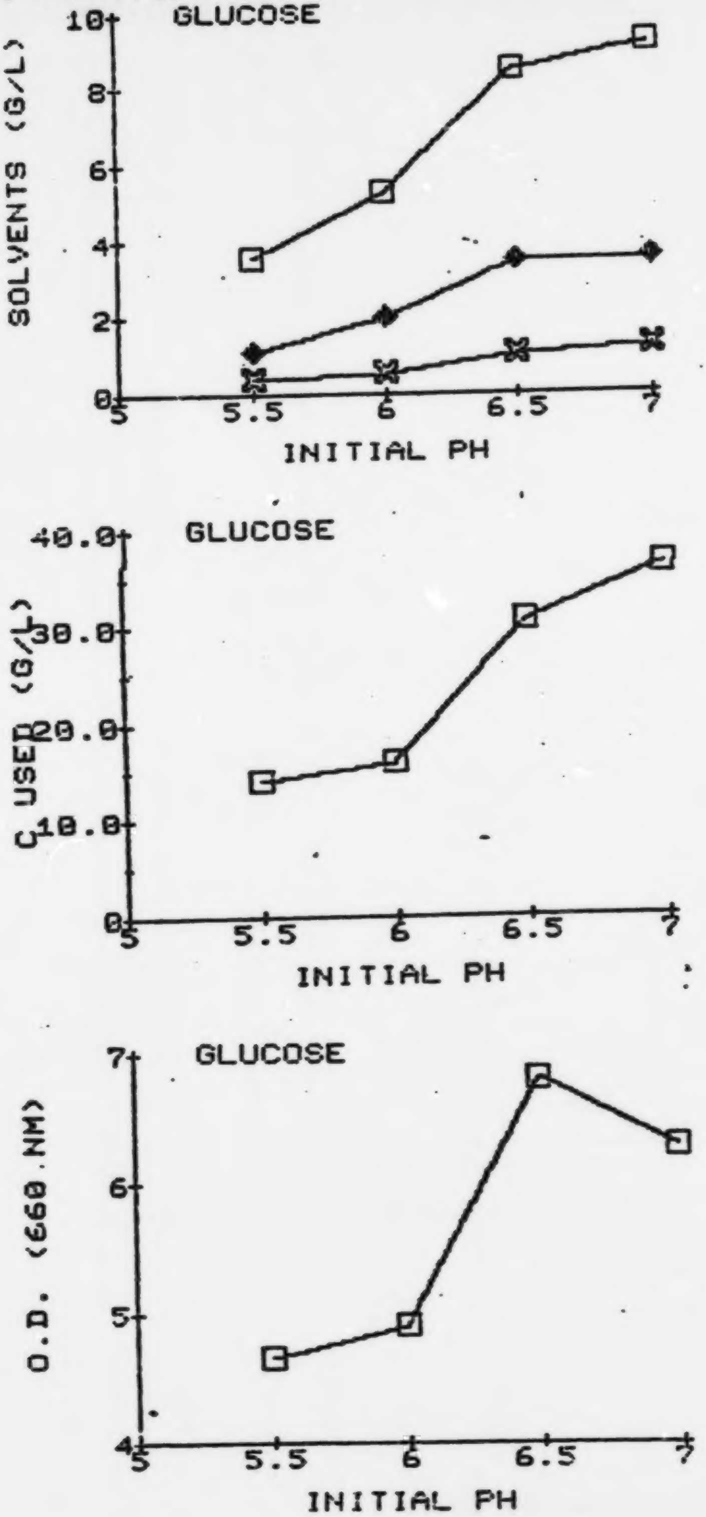


Fig. 31 The effect of initial pH on acetone-butanol fermentation of *C. acetobutylicum* grown on xylose. Solvents: □, butanol; ◆ acetone; ⊠ ethanol.

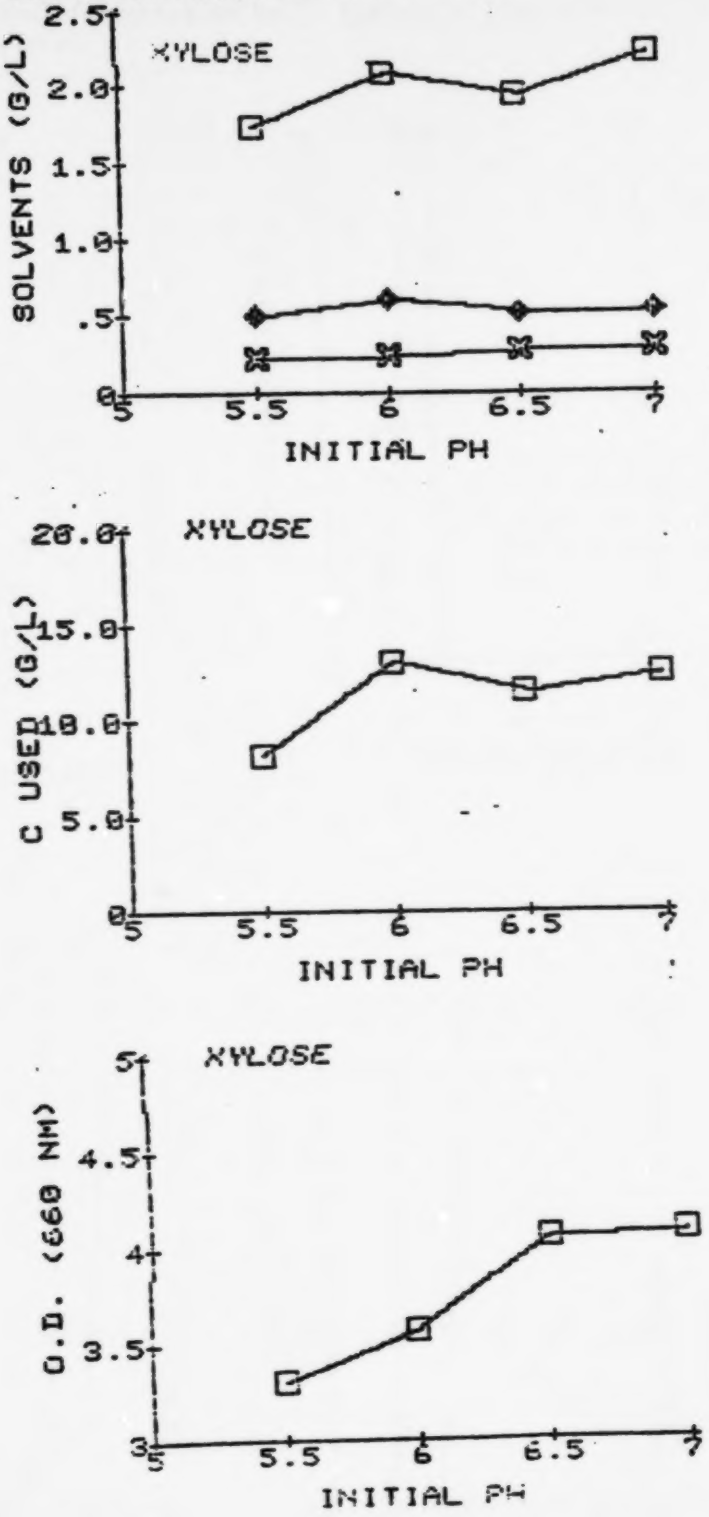


Fig. 32 The effect of acetic acid addition on acetone-butanol fermentation.
Top Left: Acetic acid added initially;
Top Right: Acetic acid added after 1 day
Solvents: □, Butanol; ◆, acetone; ⌘, ethanol.
O.D. & C used: □, ◆, corresponds to top left and top right, respectively.

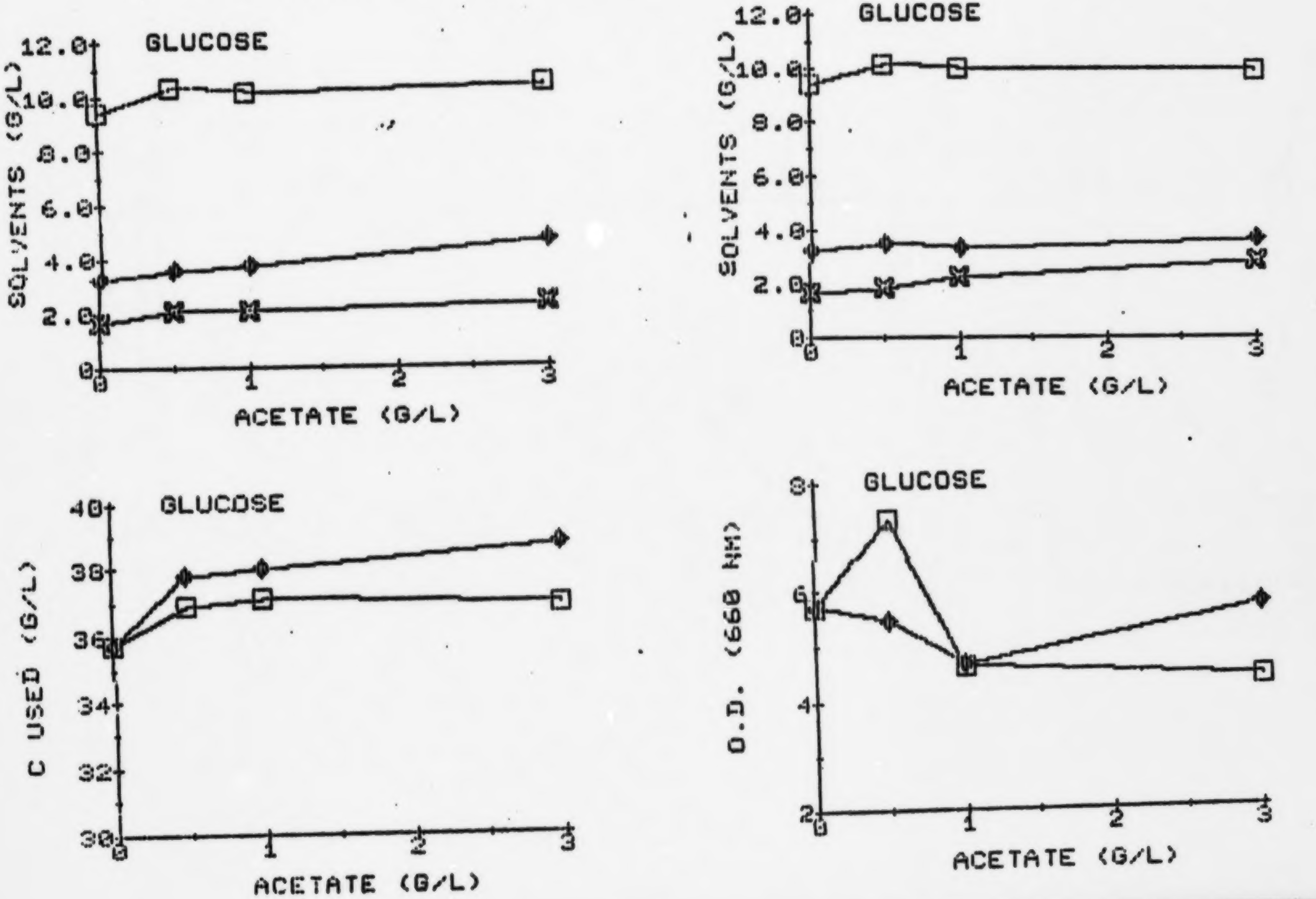


Fig. 33 The influence of acetic acid addition on acetone-butanol fermentation by *C. acetobutylicum* grown on D-xylose.

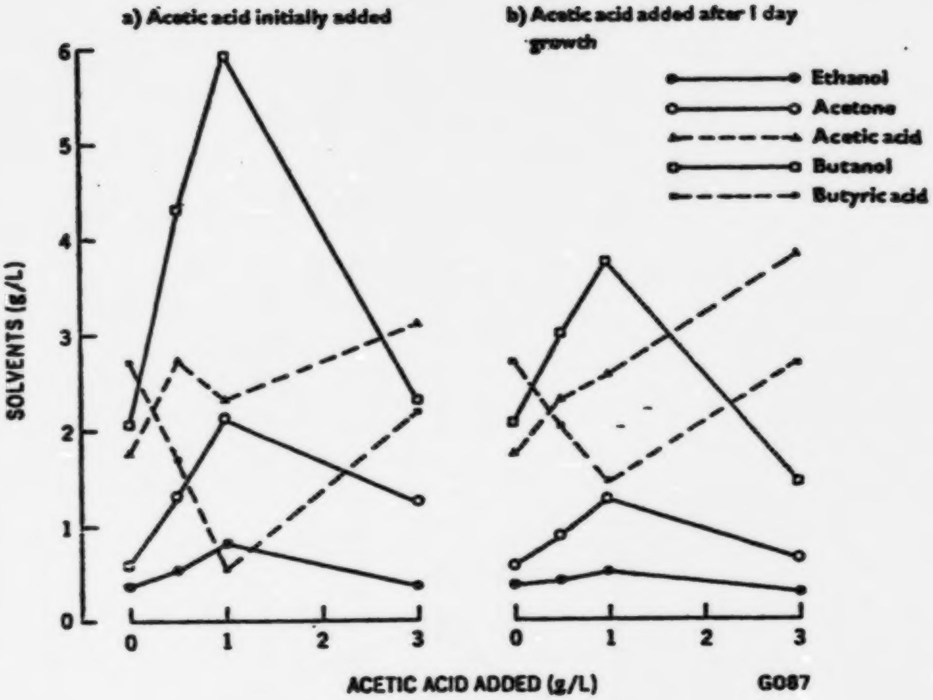


Fig. 34 The effect of acetic acid on growth and sugar utilization of C. acetobutylicum
□, acetic acid initially added;
◆, acetic acid added after 1 day growth.

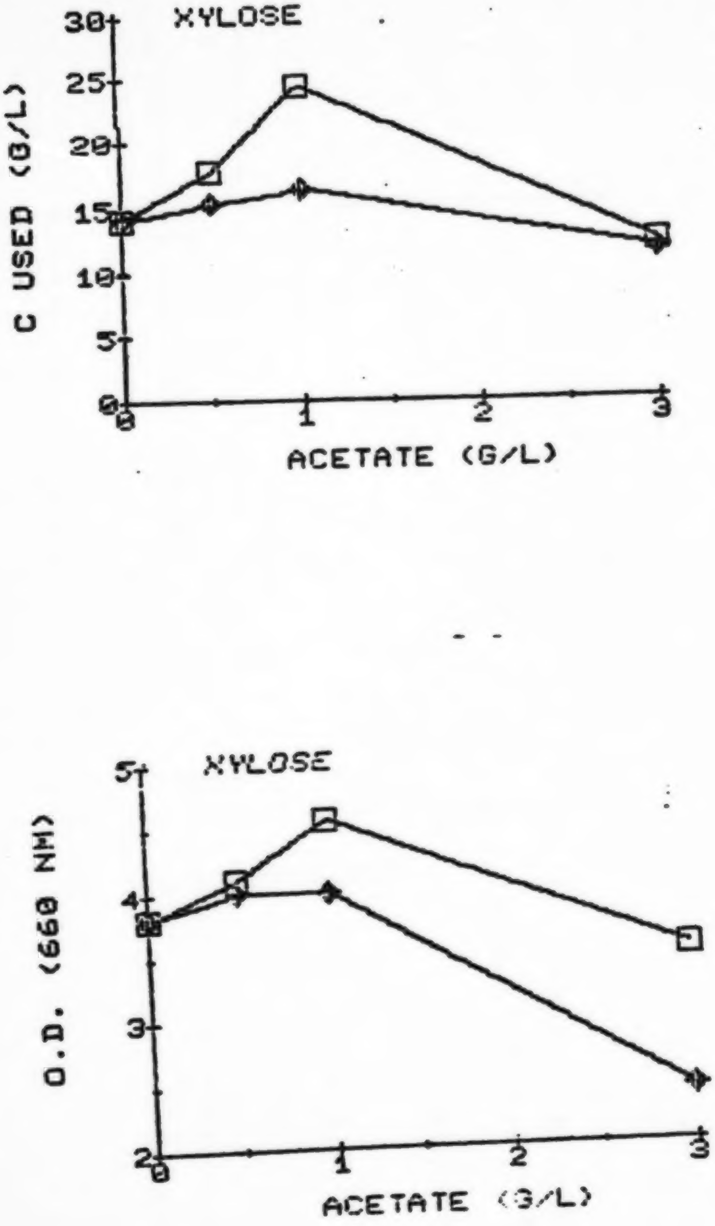


Fig. 35 The influence of butyric acid on acetone-butanol fermentation of C. acetobutylicum growth on D-glucose ,
Top left: acid initially added;
Top right: acid added after 1 day growth
Solvents: □, Butanol; ◆ acetone; ⊠, ethanol, O.D. & C used: □, ◆, as of top left & right, respectively.

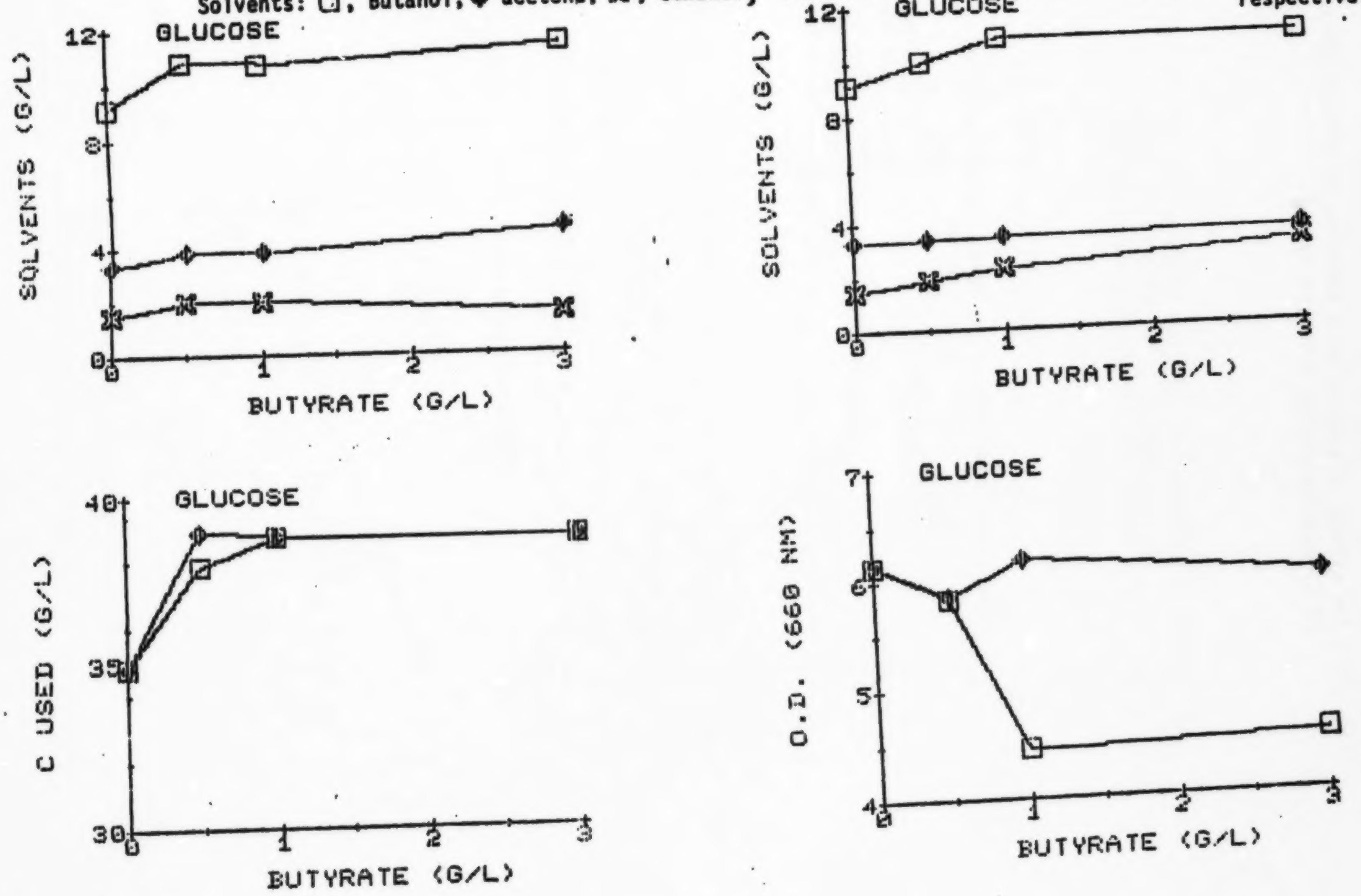


Fig. 36 The effect of butyric acid addition on solvent production by *C. acetobutylicum* grown on xylose.

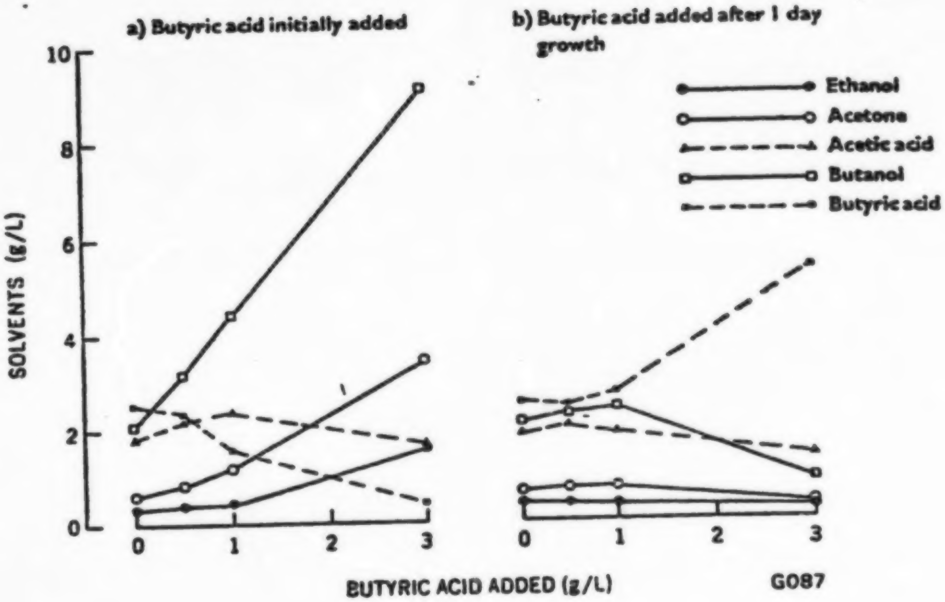
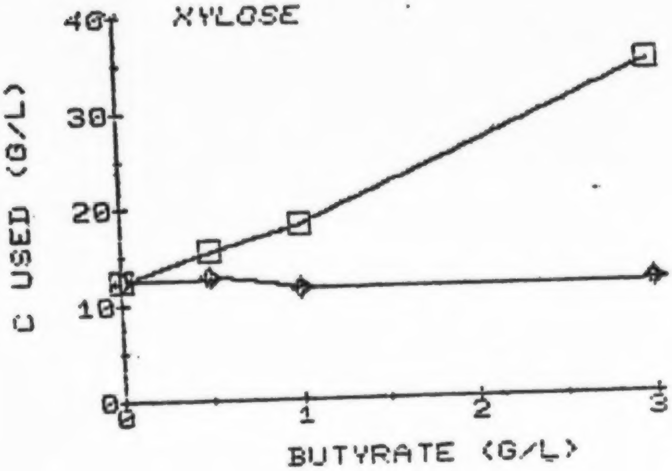
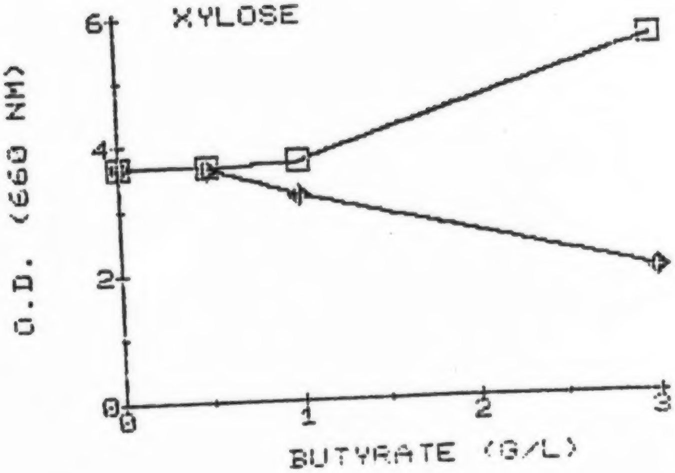


Fig. 37 The influence of butyric acid addition on growth and sugar utilization of *C. acetobutylicum*. □ , butyric acid initially added; ◇ butyric acid added after 1 day growth





POOR

QUALITY

ORIGINAL